Characterisation and selection of room temperature ionic liquids as co-solvents for redox biocatalysis.

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To my family

Abstract

The use of biocatalysis to create chiral centres for chemical pharmaceuticals is rapidly becoming a standard industrial technology. Often, the compounds of interest are poorly soluble in aqueous solvents leading to the use of organic co-solvents to deliver substrate into the environment of the biocatalyst. Unfortunately these co-solvents can impact negatively on the stability of the biocatalyst. Recently, room temperature ionic liquids have been attracting interest as potential replacements for organic solvents in such reactions as they offer lower toxicity and an increased rate of reaction. This thesis describes an original experimental study into the use of ionic liquids with biocatalytic redox reactions.

Initially, the stereospecific reduction of 6-Br-β-tetralone to its corresponding alcohol (*S*)-6-Br-β-tetralol was carried out by the yeast *Trichosporon capitatum* MY1890 and by the bacterium *Rhodococcus erythropolis* MA7213 using a range of ionic liquids chosen on the diversity of their composition. The decrease in cell viability of both types of whole cell biocatalyst upon exposure to ionic liquids was found to be intermediate to that determined for cells residing purely in fermentation media and cells residing in a two-phase mixture of media and organic solvent (toluene). For *T. capitatum* MY1890 bioconversions, the ionic liquid [Emim][TOS] gave a reaction profile comparable to that observed in the previously studied water-ethanol system, in terms of overall rate of reaction ($0.2g(prod)L^{-1}hr^{-1}$) and conversion (100% w/w). For bioconversions carried out with *R. erythropolis* MA7213, through use of 20% v/v [Emim][TOS] as a co-solvent the conversion yield doubled and a four-fold increase in initial rate was found compared to the standard ethanol co-solvent.

Subsequent flow cytometric analysis of the *R. erythropolis* MA7213 in these ionic liquids showed that the stability of the biocatalyst was increased in ionic liquids, such as [Emim][TOS] and [Oc_3MeN][BTA] accounting for the increased rates of reaction and overall conversion observed. The potential for multi parameter flow cytometry to be used as a tool for identifying biocompatible ionic

liquids was demonstrated through the improved oxidation of 1-indanone to 3,4-dihydrocoumarin by cyclo-hexanone mono-oxygenase expressed by *E. coli* TOP10 pQR239 in the presence of 20% v/v [Emim][TOS] with 30% w/w overall conversion compared to only 20% w/w overall conversion in the presence of 10% v/v ethanol.

The ability to perform isolated enzyme catalysed conversions in ionic liquids was also investigated. The asymmetric reduction of 6-Br-β-Tetralone to its corresponding (*S*) alcohol was found to be mediated by alcohol dehydrogenase isolated from *R. erythropolis* (ADH RE) and proceeded more favourably in both a miscible and immiscible ionic liquid compared to the organic solvent systems identified, with initial rate increases of more than two-fold. The ionic liquid [BMP][BTI] acting as a co-solvent (20% v/v) yielded 100% conversion of 50 gL^{-1} substrate in less than 12 hours (an increase of 25 times over the maximum yield from whole cells). This was attributed to a combined effect of increased enzyme stability in the ionic liquid and more efficient mass transfer of substrate from the ionic liquid phase to buffer compared to toluene. The asymmetric reduction of 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol was also carried out using the ADH RE and was found to be limited to 10 gL^{-1} conversion due to toxicity of the substrate on the enzyme in the presence of toluene (10% v/v). Using 10% v/v [BMP][BTI] as a co-solvent led to an enzyme half life of almost 300 hours at 30°C and complete conversion of 50gL^{-1} substrate in less than 10 hours.

The selection of a suitable ionic liquid for a particular bioconversion appears to rest primarily on biocatalyst stability, but is also dependent on solubility and mass transfer issues of substrate from the ionic liquid into the aqueous phase. The potential of a high throughput ionic liquid selection strategy based on these measurements was shown through the use of automated pipetting platforms, miniature bioreactors with online monitoring, and microwell compatible devices for flow cytometry and spectrometry for biocatalyst stability studies. The potential for ionic liquids to be employed in industrial redox bioconversions with both whole cell and isolated enzyme biocatalysts has thus been demonstrated.

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Last, but by no means least, I would like to thank my family for their unwavering support, without whom none of this would have been possible. This thesis is dedicated to them.

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Figure B.4 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the yeast *T. capitatum* MY1890 in the presence of 20% v/v [Emim][TOS]: (●) 6-Br-β-tetralone; (○) 6-Br- β-tetralol; (-) mass balance. Cultivation was performed as described in Section 2.6. Bioconversion was performed as described in Section 2.7.1 and the reaction was monitored by HPLC analysis as described in Section 2.8.1.

Figure C.1 Response of *R. erythropolis* MA7213 cells to exposure to 10% v/v Ethanol. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure C.2 Response of *R. erythropolis* MA7213 cells to exposure to 20% v/v [Bmim][BF_4]. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure C.3 Response of *R. erythropolis* MA7213 cells to exposure to 20% v/v [Emim][TOS]. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure C.4 Response of *R. erythropolis* MA7213 cells to exposure to 20% v/v [CABHEM][$MeSO_4$]. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.1 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Bmim][BF_4]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.2 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Bmim][$MDEGSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.3 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Bmim][$OcSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion

as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.4 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [CABHEM][$MeSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.5 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Emim][TOS]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.6 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Oc_3MeN][BTA]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.7 Response of *E. coli* TOP10 pQR239 cells to exposure to 10% v/v ethanol. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Figure D.8 Response of *E. coli* TOP10 pQR239 cells to exposure to 10% v/v toluene. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells.

Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Abbreviations

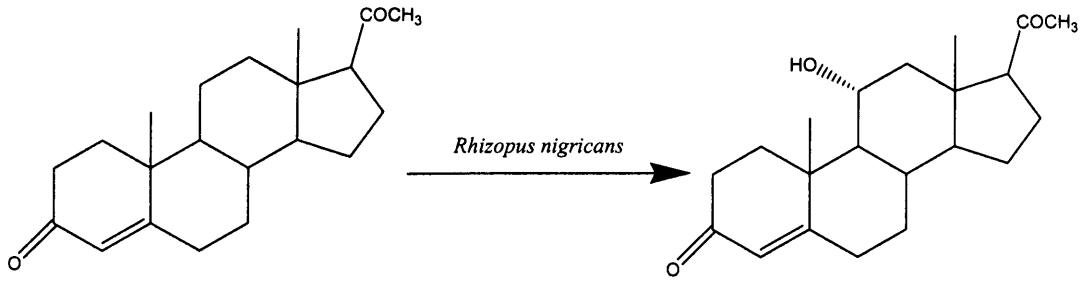
[Bmim][BF_4]	1-Butyl-3-methyl-imidazolium tetrafluoroborate
[Bmim][$MDEGSO_4$]	1-Butyl-3-methyl-imidazolium-ethylenglycolmonomethyl-ethersulfate
[Bmim][$OcSO_4$]	1-Butyl-3-methyl-imidazolium octylsulfate
[Bmim][PF_6]	1-Butyl-3-methyl-imidazolium hexafluorophosphate
[BMP][NTf2]	Butylmethylpyrrolidinium bis(trifluoromethylsulfonyl)imide
[CABHEM][$MeSO_4$]	PEG-5 Cocomonium methosulfate
EcoEng™ 21M	1-Ethyl-3-methyl-imidazolium 2(2-methoxyethoxy)ethylsulfate
EcoEng™ 212	1-Ethyl-3-methyl-imidazolium ethylsulfate
EcoEng™ 1111P	1,3-dimethyl-imidazolium dimethylphosphate
[Emim][TOS]	1-Ethyl-3-methyl-imidazolium tosylate
[EMP][ES]	1-Ethyl-3-hydroxymethyl-pyridinium ethylsulfate
[Oc_3MeN][NTf2]	Methyl-trioctyl ammonium bis(trifluoromethlylsulfonyl)imide
ADH RE	*Rhodococcus erythropolis* alcohol dehydrogenase
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
g	grams
GDH 103	glucose dehydrogenase 103
HPLC	high pressure liquid chromatography
L	litres
MTBE	methyl *tert*-butyl ether
Re	Reynolds Number
RO	reverse osmosis
rpm	revolutions per minute
THF	tetrahydrofuran
UV	ultraviolet
v/v	volume per unit volume
w/w	weight per unit weight

1 Introduction

1.1 What is Biocatalysis?

Bioconversions can be defined as 'selective enzymic modifications of defined pure compounds into defined final products'. In a bioconversion at least one of the reactants and one of the products will have structural similarity, in contrast to fermentation products where the mixture of nutrients provided has little or no structural similarity to the microorganisms grown or the result of their metabolic activities (Lilly, 1994).

The first significant industrial biotransformations were the steroid biotransformations of the 1950s, the first of which was that of Peterson and co-workers using *Rhizopus nigricans*:



Progesterone

11-α-Hydroxyprogesterone

Figure 1.1 *Rhizopus nigricans* mediated hydroxylation of progesterone to 11-α-hydroxyprogesterone (Peterson *et al*, 1952).

Prior to this process being taken up, a series of chemical reactions was required to introduce the hydroxyl group at the 11α position, with low yield and low atom efficiency. This microbiological process however yielded up to 90% product, and was very favourably looked upon in industry. This resulted in a lot of work being carried out into discovering more microorganisms capable of selectively modifying other steroids. This effort yielded two further processes very quickly; the 11β-hydroxylation of progesterone by *Curvularia lunata* and the δ1-dehydrogenation by *Corynebacterium simplex* (Nobile *et al*, 1955).

From these initial excursions, biocatalysis has become a standard technology in the fine chemicals industry, a fact illustrated by the 134 industrial bioconversions being operated around the turn of the century (Straathof *et al*, 2002). Enzyme catalysed reactions have become an essential tool in organic syntheses (Shaw *et al*, 2003). The advantages afforded by biocatalysts such as regio- and stereo-selectivity of the catalyst (illustrated by the work of Peterson *et al*, 1952) are sufficient to counter the adverse consequences of their employment- primarily the much greater cost of using biocatalysts, and so their use is increasing.

The agrochemical and pharmaceutical industries are reliant on biocatalysis to provide optically pure compounds in high quantity. Average volumetric productivity for bioconversions in the chemical industry has been rated at 15.5 $gL^{-1}hr^{-1}$ (Straathof *et al*, 2002), indicating the success of bioconversion optimisation which has occurred to improve the overall performance of biocatalysis and to overcome the negative cost aspects of employing biocatalytic

processes. Of the 134 industrial biotransformations currently being operated over 50% are in the pharmaceutical sector (Straathof *et al*, 2002).

The stereo specificity of enzyme reaction systems is seen as the primary advantage of using biocatalytic routes. Products containing multiple chiral centres are more easily produced using biocatalytic routes since the natural specificity negates the need for the multiple protection and deprotection steps required in chemical syntheses (Lye *et al*, 2002).

Further to these reactions that have already been exploited, work is currently being done on two new classes of reaction; asymmetric carbon-carbon bond formation, which can be done enzymatically through transketolase or aldolase, in order to create new chiral centres with high optical purity, and selective oxidation reactions. Whole cell systems have been developed where hydroxyl groups or oxygen can be inserted at specific positions on complex molecules with no requirement for protecting groups (Lye *et al*, 2002). Such asymmetric addition will increase the stereospecificity of enzymes and will reduce the problem of low enantiomeric yield.

1.2 Modes of biocatalysis

Bioconversions can be operated in two modes: whole cell, and isolated enzyme. In the first case the microbial culture is grown in a fermenter to a defined cell density, and the enzyme substrate (reactant) is added. After a set time period, the product is recovered from the broth. A number of challenges result from this

approach, primarily that the product must be separated from the unused broth components, and other products of the cells metabolism. Further to this, the optimum conditions for the bioconversion may not be the same as the optimum conditions for cell growth. This latter problem can be overcome by harvesting the cells and charging them into another vessel for the bioconversion (Ballard *et al*, 1983).

However, whole cell systems do offer certain critical advantages. Enzymatic redox reactions are highly efficient, and where regio- or stereoselectivity is required enzyme routes are often the best choice for an efficient process. The redox cycles in the cell require enzyme cofactors to donate or accept electrons, and these must be subsequently regenerated to their original state to catalyze another reaction, since the concentration of cofactor in the cell is finite (Endo and Koizumi, 2001).

In response to the co-factor regeneration problem robust whole cell systems have been developed for enzymatic redox reactions requiring a cofactor. The US company Eli Lilly developed the whole cell system in which a prochiral ketone is reduced to an optically active secondary alcohol, in the presence of the cofactor $NADH_2$ (Anderson *et al*, 1995).

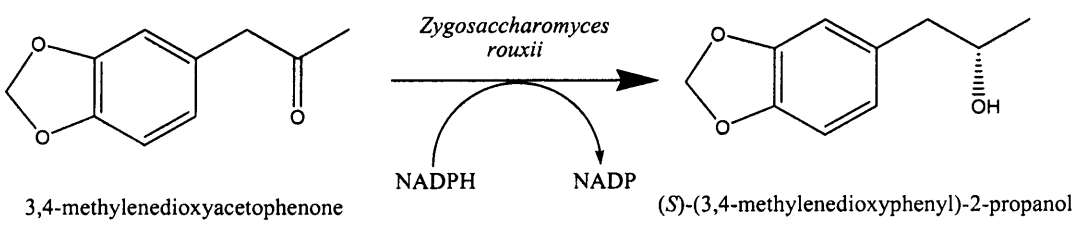


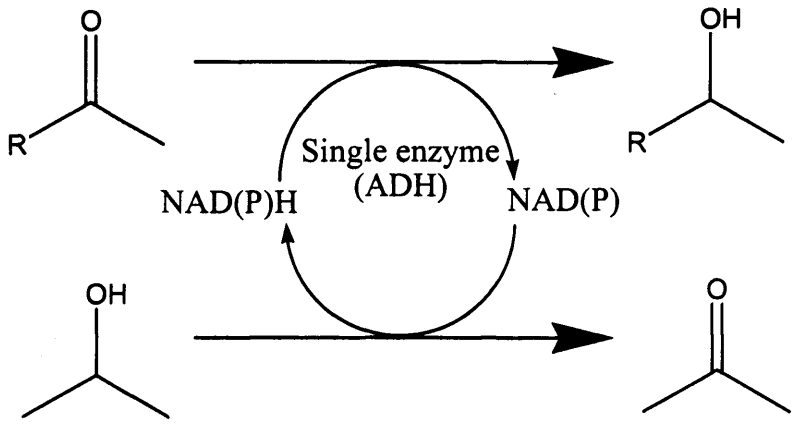
Figure 1.2 Whole cell *Zygosaccharomyces rouxii* dehydrogenase bioconversion (Anderson *et al*, 1995).

The use of isolated enzymes for bioconversions is the ideal solution for conversions that do not require cofactors. This approach eliminates the difficult task of isolating the desired product from the much more complex mixture of compounds in the case of whole cell mediated conversions, such as in the use of *Pseudomonas putida* for the conversion of aromatics to their corresponding *cis*-diols (Ballard *et al*, 1983).

Recent advances in enzymatic synthesis have led to two potential approaches for *in situ* recycling of co-factor within an isolated enzyme bioconversion. The first is a coupled substrate approach where a single enzyme is used to effect the desired reduction of, for example, a ketone to an alcohol at the expense of one co-factor molecule which is then recycled by the oxidation of, commonly, iso propyl alcohol. The second approach is a coupled enzyme approach where enzyme A effects the required reaction at the expense of a cofactor molecule and enzyme B effects the reaction of a cosubstrate recycling the cofactor. These approaches were described by Kroutil and co-workers (2004) and can be seen in Figure 1.3.

Enzyme isolation and purification was initially very difficult due to the lack of techniques for the large-scale release of enzymes from the interior of cells, so that only those enzymes that were secreted into the fermentation broth could be isolated, or the limited number of enzymes that could be released intact through chemical lysis. The development of a high pressure homogeniser however allowed industrial scale access to enzymes found in the cytoplasm, which vastly increased the number of enzymes available for use (Hetherington *et al*, 1971).

(a)



(b)

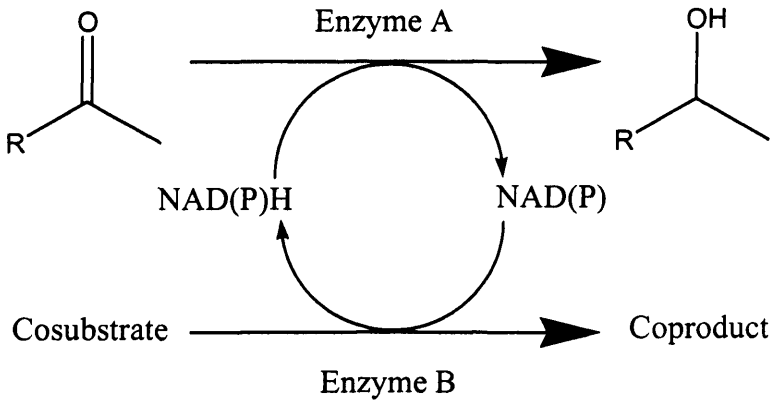


Figure 1.3 The alternatives for the regeneration of the cofactor in isolated enzyme bioconversions: (a) co-substrate (b) co-enzyme methods (Kroutil *et al*, 2004).

The cost of producing and isolating an intra-cellular enzyme is still too high to justify a single use approach (Marin-Zamora *et al*, 2006). Along with the advances in access to intra-cellular enzymes, work was being carried out to immobilise water-soluble enzymes into solid forms that could be more readily recovered and reused (Hong *et al*, 2007). Numerous techniques to immobilise catalysts were developed which included, for example, the ionic or covalent binding of the enzyme to a porous particle (Hou *et al*, 2007). Similar techniques can be applied to whole cells, by entrapping the catalyst in a porous structure akin to that used in the case of an isolated enzyme (Petre *et al*, 1999; Becerra *et al*, 2001; Trelles *et al*, 2004; Tamalampudi *et al*, 2007).

A number of process options are consequently available for bioconversions, and these decisions have formed the basis for biocatalytic processes for many years.

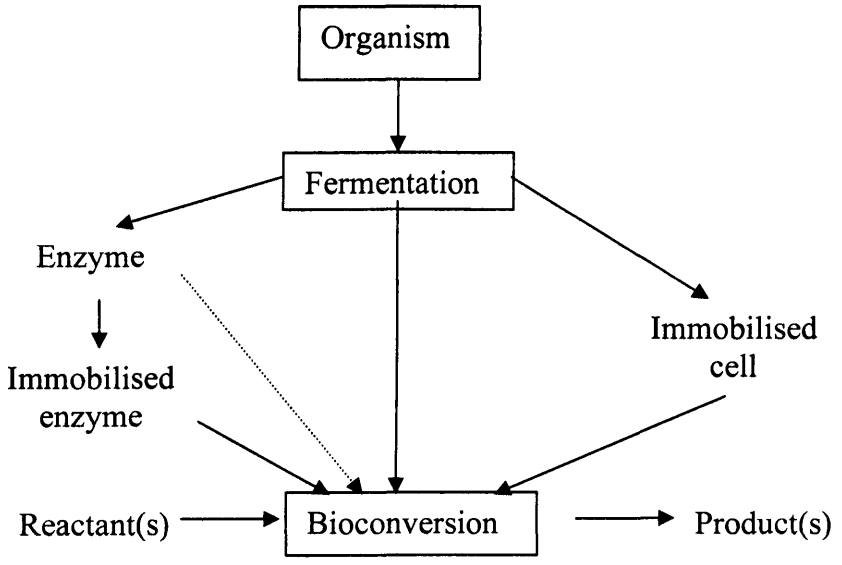


Figure 1.4 Alternative processing routes for bioconversion processes (Lilly, 1994).

1.3 Biocatalysis in pharmaceutical synthesis

A critical factor in determining the safety and efficacy of pharmaceutical drug products is the chirality of the product, so production of chiral intermediates as single enantiomers has become a critical feature of synthesis in the pharmaceutical industry (Patel, 2006). In view of the well known advantages of biocatalysis relative to chemical synthesis, i.e. the highly enantioselective and regioselective nature of enzymes, and the ability to conduct reactions at ambient temperatures and atmospheric pressure, biocatalysis is being applied to many organic syntheses (Sheldon *et al*, 2002; Patel, 2004*a*; Turner, 2004; Faber and Kroutil, 2005; Robertson and Bornscheuer, 2005; Ishige *et al*, 2005). However, the application of biocatalysis to the synthesis of pharmaceuticals will be focussed upon here.

The process for the production of the HIV-1 protease inhibitor Atazanavir (Bold *et al*, 1998) by Novartis Pharma AG (Basel, Switzerland) includes an asymmetric whole cell reduction of an aromatic ketone to chiral alcohol described in Figure 1.5. The whole cell route yielded the desired 1(*S*),2(*R*) enantiomer in 99.4% excess and over 90% yield (Patel *et al*, 2003). In contrast chemical reduction by sodium borohydride mainly produced the undesired enantiomer (Xu *et al*, 2002).

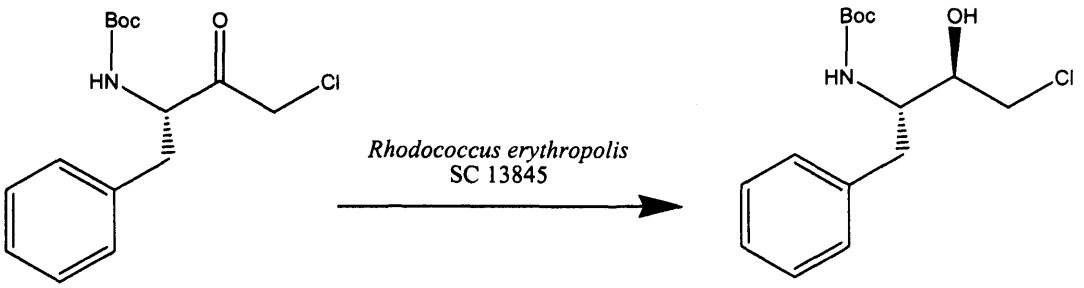


Figure 1.5 The reduction of a Boc protected ketone to its corresponding (*S*) alcohol by *R. erythropolis* SC 13845 critical in the synthesis of Atazanavir.

Bristol-Myers-Squibb developed the serotonin 5-HT_{1A} receptor modulator Buspirone (Fulton and Brogden, 1997). In this case it was shown that the two enantiomers of hydroxybuspirone were effective in treating anxiety but the (*S*) enantiomer was desired as it was cleared more slowly from the blood stream. It was found that racemic 6-acetoxybuspirone could be converted by amano acylase 30000 in 46% yield with 96% excess of the desired (*S*)-6-hydroxybuspirone (Hanson *et al*, 2005*a*). This is shown in Figure 1.6.

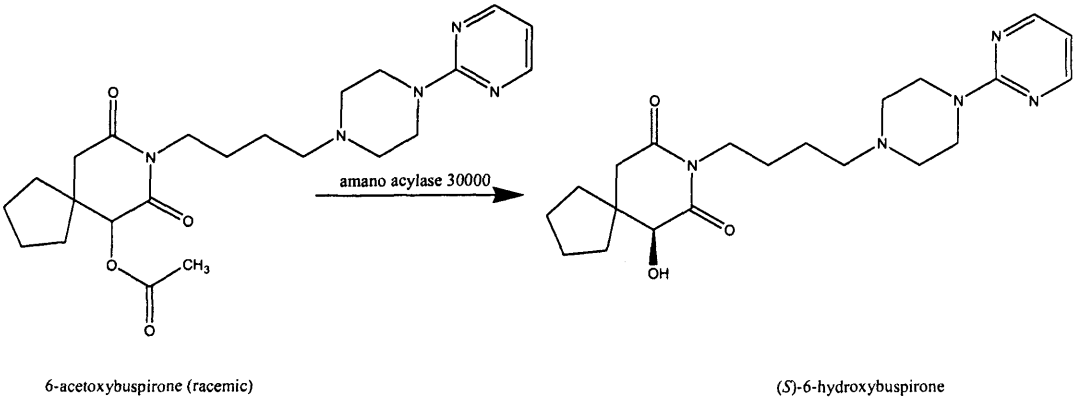


Figure 1.6 Reduction of 6-acetoxybuspirone to (*S*)-6-hydroxybuspirone by amano acylase 30000 in the synthesis of Buspirone.

A further product developed by Bristol-Myers-Squibb is a tyrosine kinase synthesised anti cancer compound requiring (*S*)-2-Cl-1-(3-chlorophenyl)ethanol as an intermediate. A screen of ~100 microbial cultures found that the desired enantiomer could be produced with ~73% excess by *Hanseula polymorpha* SC 13824 and *Rhodococcus globerulus* SC 16305. The enzyme from *H. polymorpha* was subsequently isolated and purified to yield the desired alcohol with 100% excess (Hanson *et al*, 2005*b*). The reaction is described in Figure 1.7.

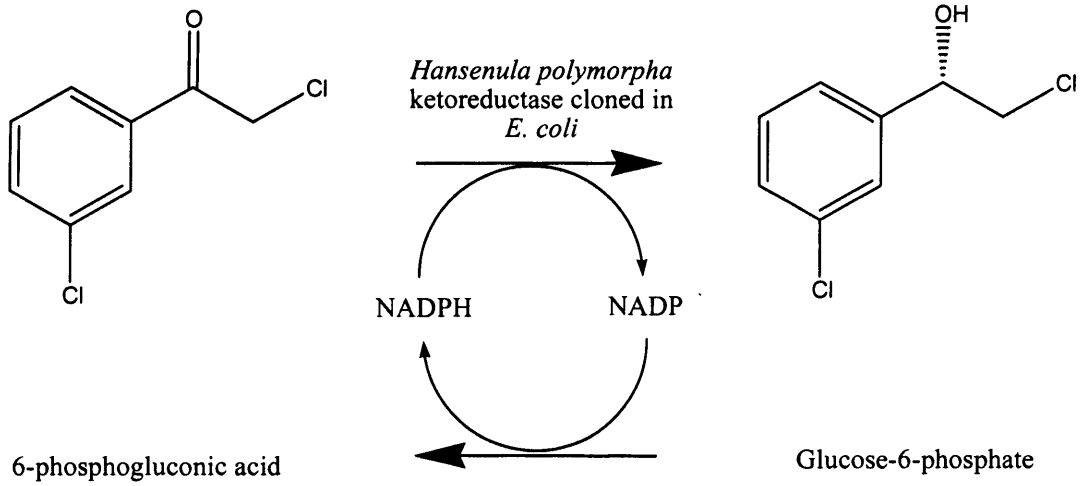


Figure 1.7 The reduction of 2-Cl-1-3-chloroacetophenone to (S)-2-Cl-(3-chlorophenyl) ethanol by a ketoreductase cloned from *H. polymorpha.*

The microbial reduction of the multi substituted aromatic ketone 2-bromo-4-fluoro acetophenone to its corresponding (*S*) alcohol is a key step in the synthesis of potential drugs for the treatment of Alzheimer's disease (Anderson *et al*, 2005). Numerous micro organisms were found through screening from the genera *Candida*, *Hansenula*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sphingomonas* and Baker's yeast that effected the reduction with a >90% yield

with the desired enantiomer in >99% excess (Patel *et al*, 2004*b*). The reaction is described in Figure 1.8.

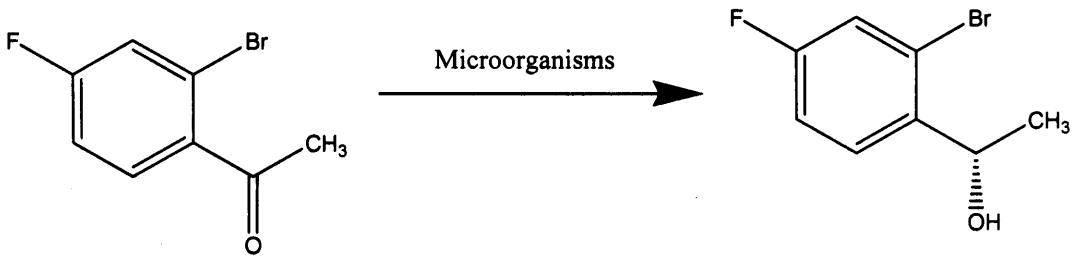


Figure 1.8 The reduction of 2-Br-4-F-acetophenone to (*S*)-2-Br-4-F-acetophenyl alcohol by various microorganisms.

Many more examples abound in the literature of the production of single enantiomers of drug intermediates in the pharmaceutical industry (Gijsen and Wong, 1995; Vicenzi *et al*, 1997; Patel *et al*, 1998; Campos *et al*, 2000; Pamies and Baeckvall, 2001; Robertson *et al*, 2004; Soni and Banerjee, 2005). Particularly, the asymmetric reduction of prochiral ketones to chiral alcohols is a critical step in the synthesis of such drug compounds (Hummel *et al*, 2003; Hanson *et al*, 2005*b*; Pollard *et al*, 2006*b*). Thus the drive towards more efficient, economic biocatalytic processes is critical to the development of more complex pharmaceutical products. Some of the recent innovations in biocatalysis to meet the industrial needs are discussed in the following sections.

1.4 Biocatalysis in unconventional media

Many of the compounds of commercial interest that have the potential to be converted by enzymes or microorganisms are poorly soluble in aqueous media,

and as a consequence biocatalysis in organic media has been established (Leon *et al*, 1998). Thus many biocatalytic steps run as biphasic systems which often comprise a first phase which is aqueous, containing the biocatalyst and a second organic phase which is comprised of the solvent and the enzyme substrate.

It has been observed that placing enzymes in anhydrous environments can adapt their functionality. In water, numerous lipases, esterases and proteases catalyse the hydrolysis of esters to their corresponding acids and alcohols, but in anhydrous solvents the same enzymes catalyse transesterification reactions when supplied with the appropriate nucleophile, in this case alcohols (Klibanov, 2001). The catalytic activity of enzymes in neat organic solvents is much lower than in aqueous systems as was demonstrated by Lee and Dordick (2002).

This is not necessarily a problematic limitation. In the cases of α-chymotrypsin and subtilisin the specificity constant of a transesterification reaction in anhydrous octane exceeds the rate constant without enzyme by 10^{11} fold; a very useful improvement. Further, in hydrous solvents activity can be comparable to those in water when coupled with other advantages of organic solvent systems, e.g. polyphenol oxidase in octanol containing 3% water retains half of its activity in water (Klibanov, 1997).

The use of organic phases in biphasic systems to remove toxic products from aqueous phases during bioconversion can have detrimental effects on whole cell biocatalysts due to their toxicity (Cruz *et al*, 2004). However a number of *Pseudomonas*, *Bacillus* and *Rhodococcus* strains have been isolated that exhibit

strong solvent tolerance (de Bont, 1998). These solvent tolerant bacteria will allow whole cell biotransformations to be carried out in previously inhospitable environments, where substrate solubility may be greatly enhanced potentially resulting in very high productivity.

1.5 Ionic liquids

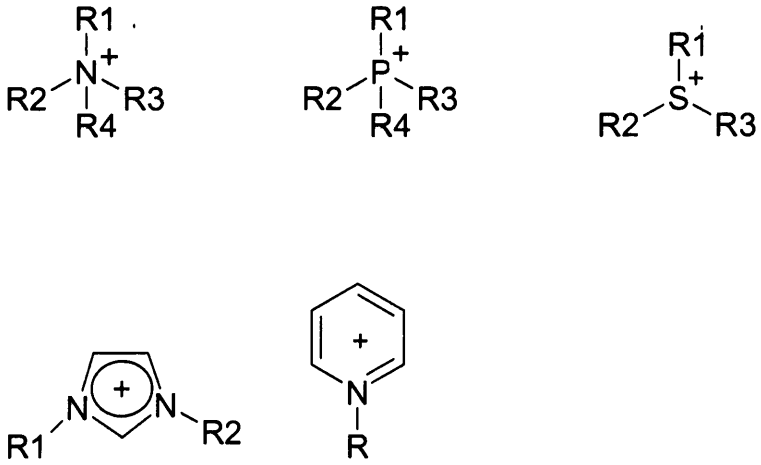
Ionic liquids are liquids composed entirely of ions, that are liquid at, or close to, room temperature, have low viscosity, and are 'easy to handle' (Sheldon, 2001). They have negligible volatility, low flammability, low toxicity, they are liquid over a wide temperature range (from -80°C to 200°C), they typically exhibit Newtonian rheology and they may be said to have tunable physicochemical properties (Roberts and Lye, 2002). This last characteristic has earned ionic liquids the accolade of 'designer solvents', since their polarity, hydrophobicity, and solvent miscibility behaviours can be tuned through the appropriate modification of the cation and anion (van Rantwijk *et al*, 2003).

Generally room temperature ionic liquids are salts of organic cations such as tetraalkylammonium or tetraalkylphosphonium, as shown in Figure 1.9. The first ionic liquid described was ethylammonium nitrate, which is liquid at room temperature, and this was reported in 1914. This was not a true ionic liquid by the modern definition as it contained 200-600ppm water. The first example of a true, modern ionic liquid was ethylmethylimidazolium tetrafluoroborate (Wilkes and Zaworotko, 1992). The corresponding hexafluorophosphate quickly followed

this (Fuller *et al*, 1994) and subsequently a plethora of room temperature ionic liquids have followed.

Ionic liquids can be prepared by the direct quaternisation of an appropriate amine or phosphine, and different anions can be added through anion exchange. However, due to the low vapour pressure of ionic liquids they cannot be purified through distillation, rather, their production must be closely monitored to ensure high purity (Sheldon, 2001).

CATIONS



ANIONS BF_4^-, PF_6^-, SbF_6^-, NO_3^-, $CF_3SO_3^-$, $(CF_3SO_3)_2N^-$, $ArSO_3^-$, $CF_3CO_2^-$, $CF_3SO_3CH_3CO_2^-$, $Al_2Cl_7^-$

Figure 1.9 Structures of ionic liquids (Sheldon, 2001).

The hydrophilicity of ionic liquids can be regulated through the choice of anion added to the cation. The ionic liquid [Bmim][BF_4] is almost completely miscible with water but the [PF_6] salt of the same cation is largely immiscible with water. Conversely, the chain length of the R groups of the cation regulates the lipophilicity of dialkylimidazolium salts and also of other ionic liquids (Sheldon, 2001).

1.6 Catalysis in ionic liquids

Applications of ionic liquids in catalysis are concentrated in two distinct directions; the first is that they replace organic solvents due to their unique properties as solvents, and the other is to replace liquid acids due to their variable acidities (DeCastro *et al*, 2000; MacFarlane *et al*, 2006). Applications of ionic liquids in place of organic solvents have taken place for example in dimerization reactions (Adams *et al*, 2000), Heck reactions (Carmichael *et al*, 1999), Friedel-Crafts acylations (Adams *et al*, 1998) and hydrogenation reactions (Suarez *et al*, 1996). Generally it has been found that the reaction rate, conversion and selectivity of the catalyst are enhanced in the ionic liquid compared to the organic solvent. Currently biphasic reaction systems run as aqueous-organic systems with water forming the bulk of the aqueous phase. This system can be improved through the use of ionic liquids in place of water. The main disadvantage of the traditional system was that catalyst ligands had to be modified in order to ensure that the catalyst was soluble in water. The protic nature of water also caused many problems which meant that water itself was

reactive with organometallic catalysts. Ionic liquids, through their inherent properties, relieve all of these problems (Zhao *et al*, 2002).

Ionic liquids have already been used in a number of catalysis reactions, and these can be categorised as hydrogen addition or rearrangement reactions, C-C and C-O cleavage reactions and C-C or C-heteroatom coupling reactions (Zhao *et al*, 2002).

Homogeneous hydrogenation has been attempted for some years in a biphasic system, with the reaction being catalyzed by transition metal complexes (Joo *et al*, 1999; Heinen *et al*, 1999). Use of traditional aqueous-organic solvent systems presented a significant problem in the necessity to modify the ligands of the complex to facilitate water solubility. The catalyst $[RuCl_2\text{-}(S)\text{-BINAP}]_2\text{-}NEt_3$ can be used to catalyze an asymmetric dehydrogenation reaction. The catalyst can be dissolved in the ionic liquid $[Bmim][BF_4]$ without any need for modification and used in a biphasic system with isopropanol. The system is capable of hydrogenating 2-arylacrylic acids with enantioselectivities higher than those obtained in homogeneous media (Monteiro *et al*, 1997).

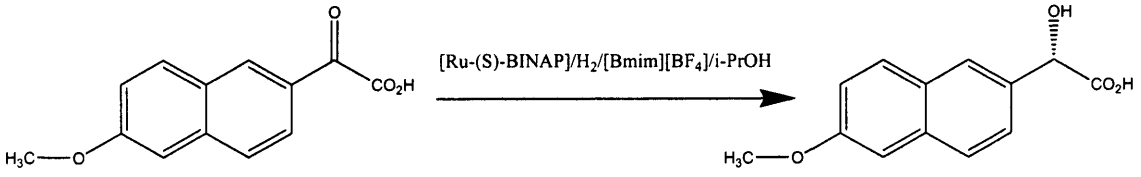


Figure 1.10 Asymmetric hydrogenation by Ruthenium catalyst in the presence of $[Bmim][BF_4]$ (Monteiro et al, 1997).

The post reaction separation is also much simpler in the ionic liquid system, and can be achieved through simple decantation. The ionic liquid phase, containing the catalyst, can be recycled without significant changes in activity or selectivity (Monteiro *et al*, 1997).

Isomerization is of great industrial importance, and has been done in the past through aqueous-organic biphasic systems with limited success; very low reaction rates were observed and this was attributed to the poor solubility in water of organic substrates. Also the protonation ability of water makes a number of the ruthenium and rhodium complex catalysts unstable in such systems. A new approach involves the use of ionic liquids based on 1-*n*-butyl-3-methylimidazolium salts as non aqueous solvents for rhodium catalysed two-phase hydrogenation, isomerisation, and hydroformylation of unsaturated substrates. It was identified that catalysts could be immobilised in these solvents liquids due to their ionic characters. [Bmim] salts of [SbF_6], [BF_4], and [$CuCl_2$] were applied to the hydrogenation of pent-1-ene using the Osborn complex and competitive isomerisation was observed in the ionic liquid (Chauvin *et al*, 1996).

Carbon-carbon bond cleavage reactions are of significance in many industries. The cracking of polyethylene is a very important process in plastics recycling, and is commonly carried out by pyrolysis, catalytic cracking with acidic materials and reactions in supercritical water (Zhao *et al*, 2002). The process has been carried out in the ionic liquid 1-ethyl-3-methyimidazolium chloride-aluminium (III) chloride yielding C_3-C_5 gaseous alkanes and branched cyclic alkanes all of which are useful feedstocks to a number of processes (Adams *et al*,

2000). The low volatility and high stability of the ionic liquid allowed the reaction to be carried out at various temperatures and it was observed that the products of the reaction differed with the temperature at which it was carried out. The product distributions were observed to be independent of the choice of cation.

The cleavage of ethers has been investigated in ionic liquids derived from 1,3-dialkylimidazolium cations and various anions such as tetrafluoroborate, hexafluorophosphate, and heptachlorodialuminate, and the system has been shown to hold many advantages over conventional molecular solvents (Green *et al*, 2000). The numerous advantages afforded by ionic liquids through their negligible vapour pressure and large liquid range make them an ideal medium in which the kinetics of ether cleavage can potentially be enhanced.

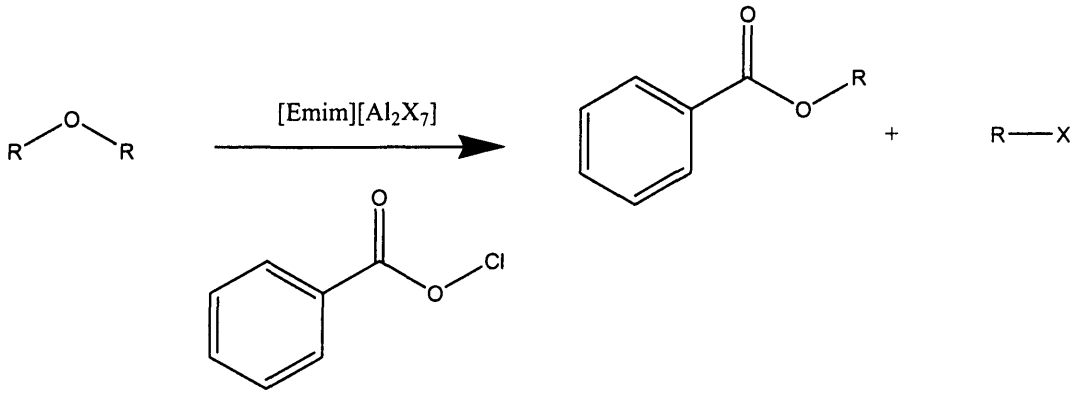


Figure 1.11 Acylative ether cleavage in the presence of an ionic liquid (Green *et al*, 2000).

Carbon-carbon coupling reactions have great commercial importance, such as in the Friedel-Crafts alkylations and acylations, and the Diels-Alder reactions. In the case of the Diels-Alder reaction, its usefulness lies in the high yields

achievable and the high stereospecificty achievable, which is of particular interest in the synthesis of natural products and physiologically active molecules (Lee, 1999). The most successful medium in use was based on lithium perchlorate-diethyl ether (LDPE) mixtures in which a greatly improved reactivity was observed due to the increased internal pressure, and the lithium ion acting as a Lewis acid. Diels-Alders reactions have been performed in a range of [Bmim] based ionic liquids where similar rate enhancements and selectivities have been observed as in LDPE. The advantages of using room temperature ionic liquids over LDPE are numerous but most significantly they have no measurable vapour pressure, can be recycled, are non-explosive, are stable over a wide temperature range and are more convenient to use (Earle *et al*, 1999).

1.7 Biocatalysis in ionic liquids

Whole cell biocatalytic systems are also capable of being run in ionic liquids. The stability of *Rhodococcus* R312 has been established in the ionic liquid [Bmim][PF_6] as an alternative to the previous toluene water biphasic system. It was found that the specific activity of the biocatalyst in the water-[Bmim][PF_6] was almost an order of magnitude greater than in the water-toluene system (Cull *et al*, 2000).

The behaviour of the cells in the two systems is of great interest. It was observed in the water-toluene system that the cells tended to aggregate and were closely associated with the liquid-liquid interface whereas the cells did not aggregate in the water-ionic liquid system. This is of great benefit down stream where product

recovery would be easier, and recycling and reuse of the ionic liquid phase, and potentially the biocatalyst, becomes more readily achievable (Cull *et al*, 2000).

Work has also been done with immobilized baker's yeast, involving the biocatalytic reduction of a ketone to its corresponding alcohol in the ionic liquid [Bmim][PF_6]. In order to prevent the inactivation of the enzyme observed in organic solvents the ionic liquid was operated as a [Bmim][PF_6]-water system in the ratio 10:1. The immiscibility of the ionic liquid with water created a biphasic system, and was seen to be the closest comparator to an organic solvent- water system. The bakers yeast was immobilised by encapsulation in calcium alginate beads and methanol was added as an energy source. The coenzyme NADPH is necessary for the reduction, but the cell is unable to recycle the cofactor in a non-aqueous environment so the reaction is limited by the initial concentration of NADPH in the cell (Howarth *et al*, 2001).

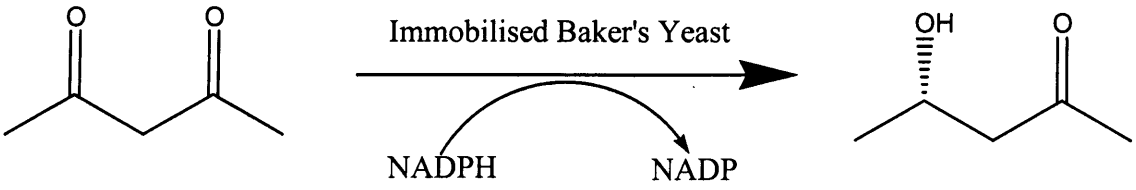


Figure 1.12 Ketone reduction by baker's yeast in the presence of [Bmim][PF_6] (Howarth *et al*, 2001).

Other work has also shown the biocompatibility of ionic liquids and their ability to support efficient whole cell bioconversion processes. In the case of the whole cell biocatalyst *L. kefir* in the presence of 3 different ionic liquids, [Bmim][PF_6], [Bmim][Tf_2N] and [OMA][Tf_2N], the cell membrane was found to be stable to

the same extent as in aqueous buffer, whereas in the presence of organic solvents such as MTBE membrane integrity was found to fall to less than 10% of the original (Pfruender *et al*, 2004). Further work by Pfruender and colleagues showed the importance of good distribution coefficients of the substrate and product between the two phases (Pfruender *et al*, 2006).

It has been known since the early 1980s that enzymes can be used in hydrophobic organic solvents, but at the price of a severely reduced reaction rate (Klibanov, 2001). However, it subsequently became clear that many lipases, as well as some proteases and acylases were so robust that they maintained their stability and activity in organic solvent environments (van Rantwijk *et al*, 2003). This much lower activity observed in organic solvents drove the emphasis towards extending the methods to ionic liquids.

The first successful isolated enzyme bioconversion in the presence of an ionic liquid was the thermolysin mediated synthesis of Z-aspartame, which was carried out in the hydrophobic ionic liquid [Bmim][PF_6] containing 5% v/v water. The resulting reaction occurred at 40% of the turnover rate in ethyl acetate (Erbeldinger *et al*, 2000).

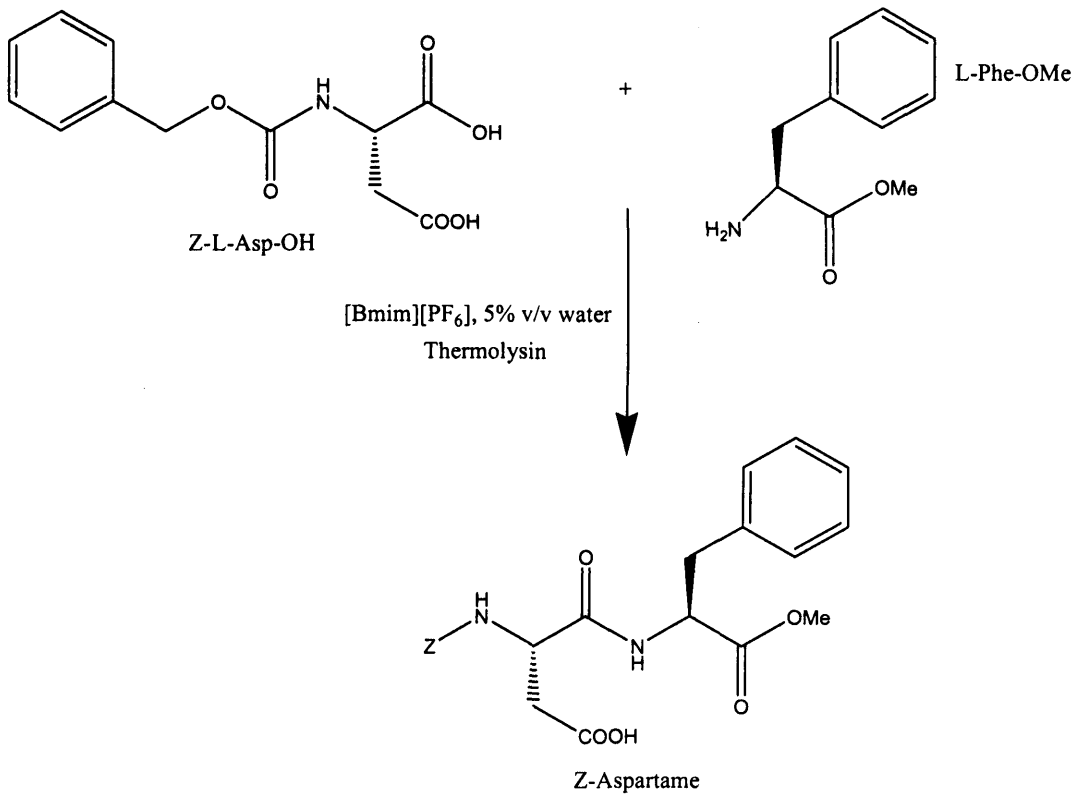


Figure 1.13 Enzymatic formation of Z-Aspartame by thermolysin in the presence of [Bmim][PF_6] and 5% v/v water (Erbeldinger *et al*, 2000).

However, the more significant result from Erbeldinger's work was that the enzyme was stabilized by the ionic liquid to a much greater degree than was the case in ethyl acetate. In organic solvents enzyme half-life is a function of the physical properties of the solvent, but the polar yet hydrophobic property of the ionic liquid in question was considered critical in stabilising the enzyme.

Lipases from *Candida antarctica* have been shown to be active in the ionic liquids [Bmim][BF_4] and also in another salt of the same cation, [Bmim][PF_6]. Reaction rates in the ionic liquids were similar or better than those observed in organic solvents (Lau *et al*, 2000). However, it was noted from this early work involving biocatalysts in ionic liquids that there was some inconsistency in the

results observed, and this was attributed to impurities resulting from the preparation of the ionic liquid. Therefore a purification step was recommended prior to use of the ionic liquid (Seddon *et al*, 2000; van Rantwijk *et al*, 2003).

The enzyme α-chymotrypsin mediates the trans-esterification of *N*-acetyl-L-amino acid esters, and the activity of this enzyme has been investigated in the presence of five different ionic liquids based on dialkylimidazolium and quaternary ammonium cations associated with perfluorinated and bis (trifluoromethyl) sulfonyl amide anions at 2% v/v water content at 50°C (Lozano *et al*, 2001; Laszlo and Compton 2001; van Rantwijk *et al*, 2003). The polarity of the ionic liquid environment was found to be sufficient in [Bmim][PF_6] and [Omim][PF_6] to negate the requirement of water, which was needed to facilitate the reaction. It was found that in these liquids non polar sCO_2 was a suitable alternative (Lozano *et al*, 2001).

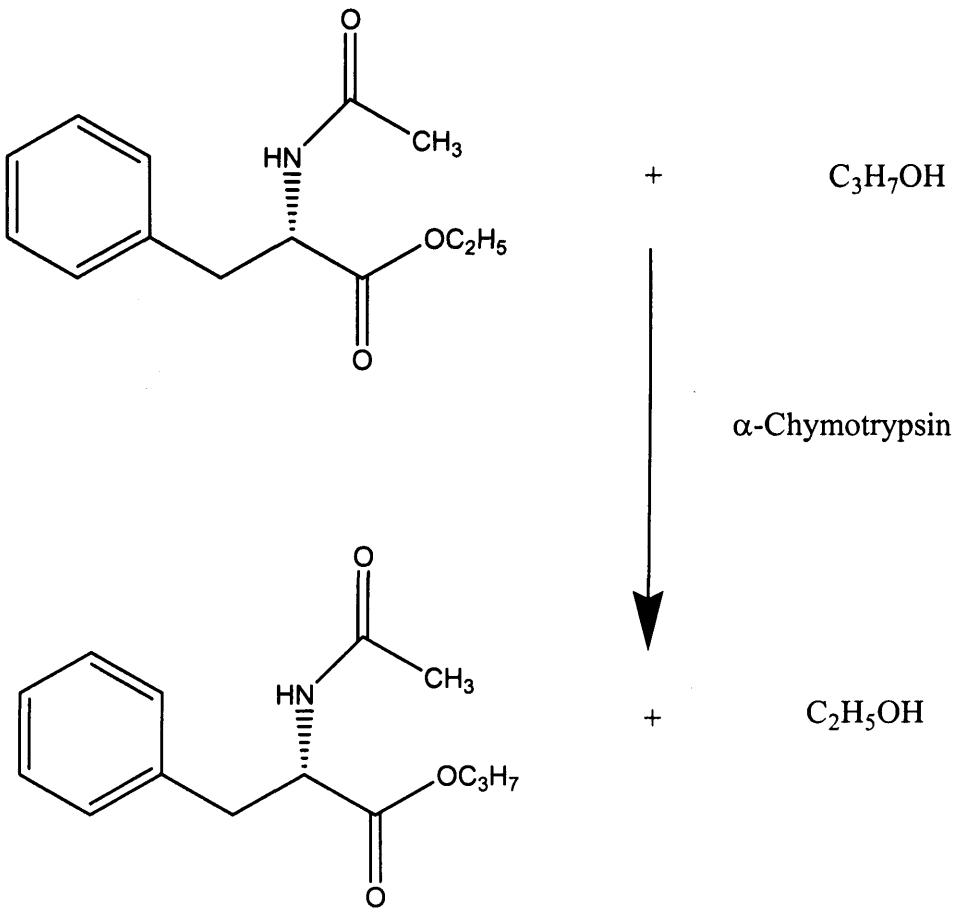


Figure 1.14 α-Chymotrypsin mediated transesterification in the presence of [Bmim][PF_6] (Lozano *et al*, 2001).

Much of the work in this area thus far has focused upon isolated enzymes, particularly lipases (Park and Kazlauskas, 2001; Kaar *et al*, 2003; Zhao, 2005), with relatively few involving oxidations (Hinckley *et al*, 2002; Laszlo and Compton, 2002) or reductions (Eckstein *et al*, 2004). The work of Eckstein and co-workers involved the enantioselective reduction of 2-octanone by alcohol dehydrogenase (ADH) isolated from *Lactobacillus brevis* in the presence of [Bmim][$(CF_3SO_2)_2N$]. However the initial ketone concentration in this study was low at < 1 gL^{-1}. Where co-factor recycling has previously been employed in the case of redox bioconversions, it was by the addition of co-substrate. The

enantioselective reduction of prochiral ketones to alcohols is an important reaction in the synthesis of pharmaceuticals (Patel *et al*, 2004*a*), but no other examples of ketone reductions by isolated enzymes in the presence of ionic liquids exist in the literature at the present time.

For biocatalytic applications ionic liquids have been shown to promote improved enzyme stability. The lipase Novozym 435 was shown to retain almost 3 times the retained activity observed in the presence of hexane after 24 hours incubation in the ionic liquid [MMEP][CH_3CO_2] and twice as much in [Bmim][CH_3CO_2] (Zhao, 2005). The activity of thermolysin was retained after incubation in [Bmim][PF_6] for 144 hours whereas almost half of the original activity was lost after similar incubation in ethyl acetate (Erbeldinger *at al*, 2000). An esterase isolated from *Bacillus stearothermophilus* was found to be greatly stabilized in the ionic liquid [Bmim][PF_6] with a half life of >240 hours which was a 30 fold increase over hexane and 3 times that in methyl tert-butyl ether (MTBE) (Persson and Bornscheuer, 2003).

1.8 Multiparameter flow cytometry

The response of whole cells to their environment has traditionally been assessed by techniques such as manual counts of colony forming units (Quiros *et al*, 2007). These measures would therefore count those cells that are capable of dividing on the agar surface to which they are attached, but would not count cells that are "viable but unculturable" (Hewitt and Nebe von Caron 2001, Sachidanandham *et al*, 2005). This means cells that are capable of metabolic

activity but are unable to divide. Multi parameter flow cytometry is an emerging method capable of detecting such subtle changes in the cellular state of bacteria.

A schematic of the operation of the flow cytometer is shown in Figure 1.15. The sample is carried in a fluid known as the sheath fluid, which regulates the system pressure and flow rate of material through it. The sample enters the flow cell where the stream is focussed to approximately a single cell flow by a focussing aperture. Here the sample is exposed to laser light at a wavelength of 488 nm. Scattered light from this exposure is detected at two angles: forward scatter in the plane of the light and side scatter at right angles to that plane. Emitted light from the fluorochromes is also detected at right angles to the plane of the laser. The light goes through a series of filters with an increasing wavelength cut off. Each of these reflects the light in their respective wave band to a photo multiplier tube that leads to a detector. By monitoring shifts in the intensity of reflected light, changes in cell characteristics can be determined.

Flow cytometry analysis of a cell population can reveal, at a cell by cell level, the physiological condition of a cell population such as cell viability, cell density, function or ion density. The parameters under investigation can be tuned according to the cell line by employing appropriate dyes that, upon excitation by a laser, emit at different wavelengths to allow analysis of multiple parameters per cell population in a single run.

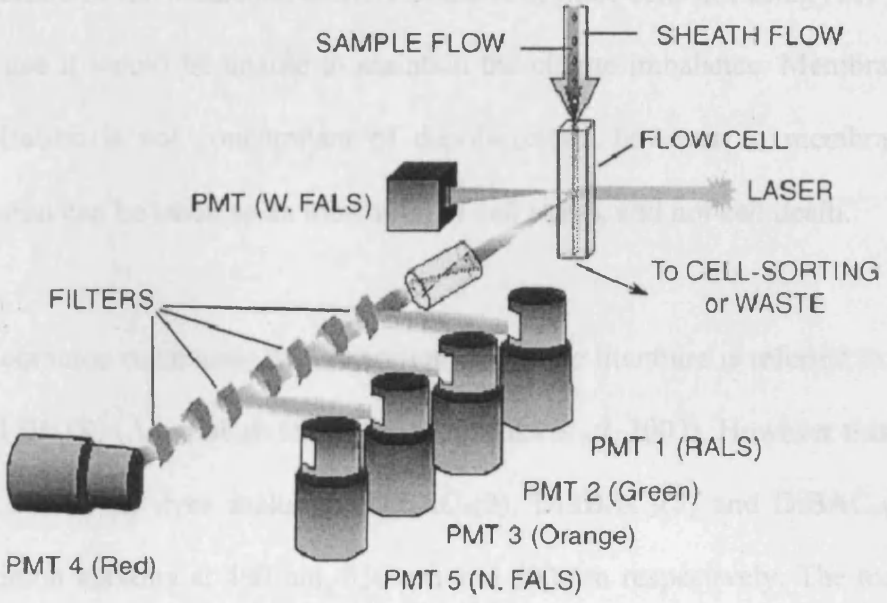


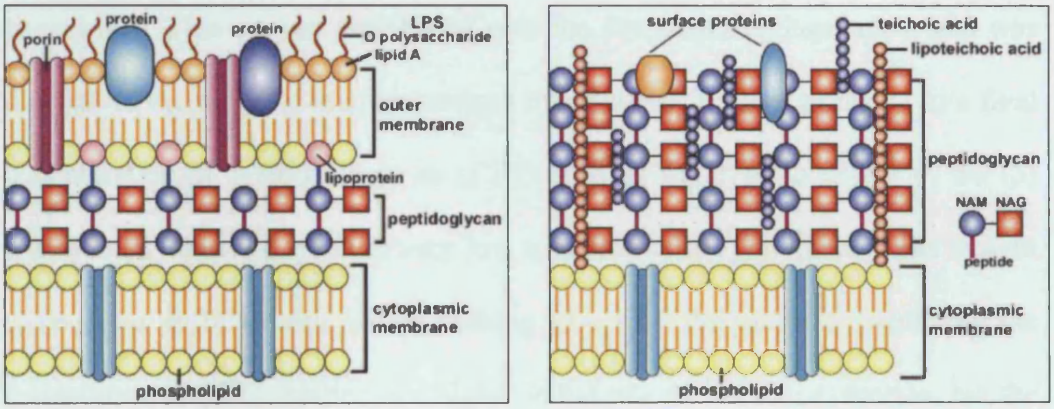
Figure 1.15: schematic describing the operation of a flow cytometer (Hewitt and Nebe von Caron, 2004).

Flow cytometry is built on the premise that a cell population can be identified through a characteristic size and "granularity"- determined through forward and side scattering of the laser in the system (Vale-Silva and Buchta, 2006). By "gating" this characteristic area and determining base values of fluorescence for each of the dyes on those events, changes can readily be identified by shifts in these fluorescence intensities.

In this work, two parameters will be determined: membrane potential and cell death. Membrane potential is achieved through the maintenance of a charge balance across the membrane by potassium/ sodium ion channels. The use of ATP by the cell causes 3 sodium ions to be pumped out of the cell and two potassium ions simultaneously pumped in resulting in a potential difference across the membrane. The maintenance of this potential difference can be taken

as an indication of the metabolic activity of the cell, since cells not using ATP or unable to use it would be unable to maintain the charge imbalance. Membrane permeabilisation is not concomitant of depolarization however so membrane depolarisation can be taken as an indication of cell stress, and not cell death.

The most common membrane depolarisation dye in the literature is referred to as bis-oxonol (BOX) (Amanullah *et al*, 2003; Onyeaka *et al*, 2003). However this is actually a family of dyes including $DiBAC_4(3)$, $DiSBAC_2(3)$ and $DiBAC_4(5)$ with excitation maxima at 490 nm, 530 nm and 590 nm respectively. The most commonly used is $DiBAC_4(3)$ but all of them operate in the same manner. The dyes enter depolarized cells and bind to intracellular proteins or membranes and exhibit enhanced fluorescence and red spectral shifts. Increased depolarization leads to more influx of the dye and thus an increase in fluorescence. However the BOX family of dyes are not suitable for use with gram positive bacteria due to the presence of the peptido glycan cell wall (Figure 1.16(b)). The dye $DiOC_6(3)$ was identified as suitable for determining membrane potential of gram positive bacteria (da Silva *et al*, 2005, Reis *et al*, 2005).



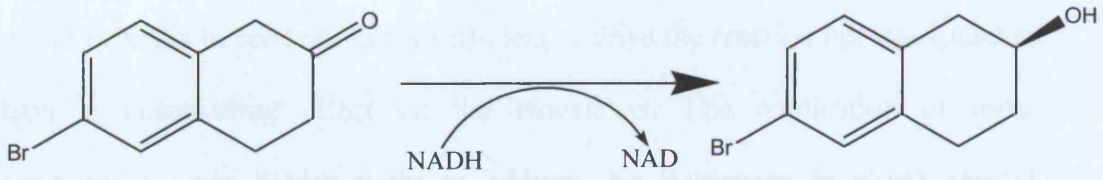
(a) (b)

Figure 1.16 Graphical depiction of the cell wall of (a) a gram negative bacterium and (b) a gram positive bacterium. Images reproduced from http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/u1fig11.html

1.9 The model systems used in this work

1.9.1 Reduction of 6-Br-β-tetralone to (S)-6-Br-β-tetralol

Work at the Merck Research Laboratories, NJ, USA identified, through a microbial screening program, the yeast *Trichosporon capitatum* as a suitable biocatalyst for the asymmetric bioreduction of 6-Br-β-tetralone to its corresponding (*S*)-alcohol, (*S*)-6-Br-β-tetralol (Reddy *et al*, 1996), shown in Figure 1.17.



6-Br-Tetralone (S)-6-Br-Tetralol

Figure 1.17 Asymmetric reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol mediated by the yeast *T. capitatum* MY1890 (Reddy *et al*, 1996) and *R erythropolis* MA7213 (Stahl *et al*, 1997).

An aqueous system yields a 71.4% enantiomeric excess (ee) of the (*S*) enantiomer. This ee was established with the *Trichosporon capitatum*, and was the best of the range of microorganisms investigated. By using ethanol to a final concentration of 5-10% v/v an ee of 93% can be achieved in favour of the (*S*) enantiomer. This is due to the very low solubility of the β-tetralone. The system is operated at 28°C with orbital shaking to agitate the mixture. Increasing the temperature to 37°C resulted in a higher initial rate of tetralol production, but the final titer of tetralol was lower than when operating at 28°C. Further screening work identified the gram positive bacterium *Rhodococcus erythropolis* MA7213 as another potential biocatalyst for this bioconversion (Stahl *et al*, 1997).

1.9.2 Reduction of 4'-Br-2,2,2-trifluoroacetophenone to (R)-4'-Br-2,2,2-trifluoroacetophenyl alcohol

Reduction of multi-substituted pro-chiral acetophenones for pharmaceutical synthesis has been previously reported with whole cell biocatalysis (Patel et al, 2004) and by isolated enzymes (Pollard *et al*, 2006*b*). In these cases the acetophenones were either highly soluble in aqueous media (Patel *et al*, 2004) or the reaction conditions could be manipulated so that the substrate was readily converted by the biocatalyst of choice. In the particular case of the reduction of 4'-Br-2,2,2-trifluoroacetophenone (Figure 1.18) the substrate is soluble in aqueous media to concentrations sufficient to drive the reaction but was found to have a deactivating effect on the biocatalyst. The application of room temperature ionic liquids seeks to address this bottleneck in chiral alcohol synthesis.

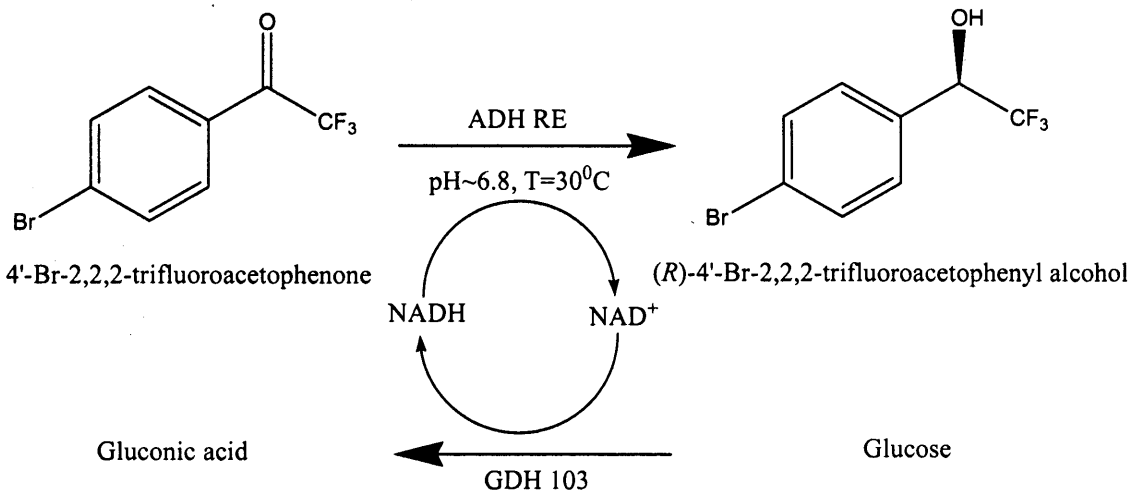


Figure 1.18 The asymmetric reduction of 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4-Br-2,2,2-trifluoroacetophenyl alcohol by alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) and co-factor recycling by the glucose dehydrogenase 103 (GDH 103) mediated oxidation of glucose.

1.9.3 Oxidation of 1-indanone to 3,4-dihydrocoumarin

The Baeyer-Villiger oxidation of 1-indanone to 3,4-dihydrocoumarin is mediated by the gram negative bacterium *E. coli* TOP10 pQR239 (Gutierrez *et al*, 2003). The pQR239 plasmid confers cyclohexanone mono-oxygenase (CHMO) activity on the *E. coli* TOP10 host isolated from *Acetinobacter caloaceticus*, as well as ampicillin resistance useful as a selection marker (Doig *et al*, 2001). Literature data has shown the bioconversion shown in Figure 1.17 proceeds with "minimum conversion" in the presence of 10% v/v ethanol (Gutierrez *et al*, 2003). The potential for ionic liquids to be used to achieve a greater conversion is investigated here through the use of flow cytometry to identify potential biocompatible ionic liquids.

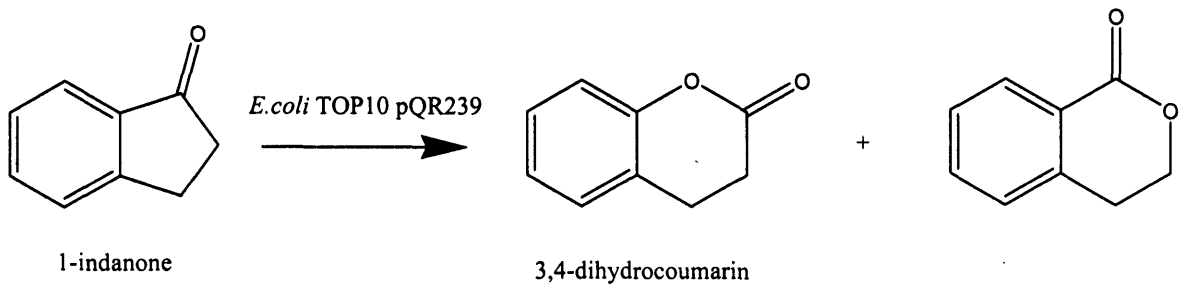


Figure 1.19 The CHMO mediated oxidation of 1-indanone to 3,4-dihydrocoumarin by *E. coli* TOP10 pQR239.

1.10 Aims and objectives

The application of biocatalysis in pharmaceutical synthesis today is dependent on the ability to rapidly screen biocatalysts and process conditions to overcome the relatively fast development of comparable chemical processes to produce the desired chiral centre (Pollard and Woodley, 2007). Biocatalyst screening is relatively simple compared to process screening as it is most often dependent on the ability of a given biocatalyst to produce a particular optically active product in sufficient excess. This is usually a more important consideration than conversion or rate of reaction as these are more readily manipulated through engineering parameters such as mixing conditions, co-solvent fraction and substrate feeding rates. However, as mentioned previously, the propensity for compounds of pharmaceutical interest to be poorly aqueous soluble makes co-solvents a critical component of process screening, in order to regulate the supply of substrate to the environment of the biocatalyst and remove product to drive the reaction. In the case of organic co-solvents the log P scale exists to identify solvents more likely to support a biocatalyst in it's active form, but no such scale

exists for ionic liquids. The primary aim of this thesis is to identify the factors critical in selecting a co-solvent for biocatalysis and to rank these factors in order of their priority. The specific objectives of this study are summarised below:

- The reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol mediated by the whole cells *Trichosporon capitatum* MY1890 and *Rhodococcus erythropolis* MA7213 will be used to determine the feasibility of ionic liquids as co-solvents for biocatalytic reduction. The effect of the ionic liquids on the viability of the whole cell biocatalysts will be determined and the effects of these changes in viability on the bioconversion efficiency.
- The potential for multi-parameter flow cytometry to be used as a tool for identifying biocompatible ionic liquids will be assessed. Two strains will be investigated: the gram positive bacterium *R. erythropolis* MA7213 and the gram negative bacterium *Escherichia coli* TOP10 pQR239. The selection of ionic liquids will be subsequently tested through the oxidative bioconversion of 1-indanone to 3,4-dihydrocoumarin mediated by the CHMO expressing *E. coli* TOP10 pQR239.
- Further to the use of whole cells for Redox bioconversions, the potential for ionic liquids to be used as co-solvents in the isolated enzyme mediated reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol and 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol with in situ co-factor recycling will be assessed. The effects of ionic liquids on the stability and activity of the enzyme will be investigated and subsequent effects on reaction kinetics.

- A conceptual framework for the selection of ionic liquid co-solvents for biocatalytic applications will be presented. The potential for this system to be automated and the units integrated will be assessed.

Finally, as part of the Engineering Doctorate requirements of the University of London, a brief appraisal of the Bioprocess Validation and Bioprocess Management consequences of the research will be presented.

2 Materials and Methods

2.1 Chemicals and microorganisms

All chemicals were obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK), or from Merck KGaA (Darmstadt, Germany), and were of the highest purity available. The 6-Br-β-tetralone, 6-Br-β-tetralol, *T. capitatum* MY1890 and *R. erythropolis* MA7213 were kindly provided by Merck Research Laboratories, Merck & Co. Inc. (Rahway, NJ, USA). The *E. coli* TOP10 pQR239 was kindly provided by Dr J. Ward, Depart of Biochemistry, University College London, UK. Ionic liquids were obtained from Solvent Innovation (Cologne, Germany) and were of >98% purity. All enzymes were procured from Biocatalytics Inc (California, USA).

2.2 Ionic liquid preparation

The hydrophobic ionic liquids were washed with water to remove any soluble impurities for whole cell reactions. The removal of, particularly acid, impurities was required in order to operate 6-Br-β-tetralone reactions (Chapter 3) between pH 6.5 to 7.0 in the aqueous phase. [Bmim][PF_6] was washed with 10 equal volumes of Reverse Osmosis (RO) water, with the pH of the final wash being ~6.5. [Oc_3MeN][NTf2] was washed with 5 equal volumes of RO water, with a final pH of the wash solution close to 7. The hydrophilic ionic liquids were used as supplied.

2.3 Quantification of substrate solubility

500 mg of substrate was weighed into a 1.5 mL HPLC vial and 1 mL of the appropriate solvent was added. These were then shaken at 1400 rpm at 30°C on a thermomixer (Eppendorf) for 24 hours. After 24 hours the samples were removed and allowed to settle before 50 μL was taken and diluted into 950 μL acetonitrile and assayed for substrate content by reverse phase HPLC (Section 2.8.1). Aqueous solubility curves were generated by taking the appropriate amount of these samples and adding to 100 mM KH_2PO_4 buffer (pH~7.0) to a total volume of 1 mL. The vials were returned to identical conditions as above and allowed to equilibrate for a further 24 hours. The samples were then allowed to settle before 50 μL was taken and diluted into 450 μL acetonitrile in a dead end filter vial to ensure no solids proceeded to solute analysis.

2.4 Quantification of substrate extraction

500 μL of ionic liquid was saturated with substrate as described in Section 2.3 and undissolved solid removed using a dead end filtration vial. 500 μl of various pure organic solvents were then added and the mixture returned to the thermomixer and allowed to mix for 3 hours at 1400rpm. For immiscible solvents, a sample of both phases was taken, dissolved in acetonitrile and the substrate concentration was determined by reverse phase HPLC analysis (Section 2.8.1). Extraction efficiency was subsequently determined as the percentage of substrate transferred to the organic solvent phase.

2.5 Enzyme screening

A total of 66 commercially available keto-reductase preparations were used each at a concentration of 2 gL^{-1} in 100 mM potassium phosphate dibasic buffer containing 1.2 molar equivalents of NADH or NADPH based on the co-factor dependency of the enzyme. Substrate (10 gL^{-1} final concentration) was delivered into the reaction in 10% v/v toluene. After 5 hours incubation at 30°C reactions were sampled into acetonitrile (to a 1/20 dilution) for reverse phase HPLC analysis (Section 2.8.1), then dried down under nitrogen and resuspended in methanol for chiral HPLC analysis (Section 2.8.2).

2.6 Microbial cultivation

T. capitatum MY1890. A frozen suspension of cells stored at -80°C in 25% v/v (final) glycerol was thawed at room temperature and used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of broth comprising 30 gL^{-1} glycerol, 25 gL^{-1} hysoy peptone, and 20 gL^{-1} yeast extract. This had been autoclaved at 121°C for 15 minutes prior to use. The culture was aerobically incubated at 28°C on an orbital shaker at 220 rpm. Bioconversion was initiated after 36 hours growth.

R. erythropolis MA7213. A frozen suspension of cells stored at -80°C in 25% v/v (final) glycerol was thawed at room temperature and was used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of autoclaved (121°C for 15 minutes) Tryptic Soy Broth (TSB, 30 gL^{-1}). The culture was aerobically incubated at 30°C on an orbital shaker at 200 rpm. Bioconversion was initiated after 20 hours growth.

E. coli TOP10 pQR239: a frozen suspension of cells stored at -80°C in 25% v/v (final) glycerol was thawed at room temperature and was used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of broth containing 10 gL^{-1} soya bean peptone, 10 gL^{-1} glycerol, 10 gL^{-1} sodium chloride and 25 gL^{-1} yeast extract. This was autoclaved (121°C for 15 minutes) and prior to use 400 mgL^{-1} ampicillin was added. The culture was aerobically incubated at 37°C on an orbital shaker at 200 rpm. After 3 hours cultivation 2.6 gL^{-1} L(+)-arabinose was added as an inducer of CHMO expression. Bioconversion was initiated after a further 4 hours growth.

2.7 Bioconversion kinetics

2.7.1 Whole cell bioconversions

After the appropriate cultivation time for each microorganism the 'standard' (or control) bioconversion was initiated by the addition of 5 mL of an ethanol solution directly to the fermentation flask. In the case of *T. capitatum* MY1890 (Reddy *et al*, 1996), this contained 55 mg of 6-Br-β-tetralone while in the case of *R. erythropolis* MA7213 it contained 22 mg of 6-Br-β-tetralone. Where ionic liquids were employed 20 mg of 6-Br-β-tetralone in the case of *T. capitatum* MY1890 and 10 mg in the case of *R. erythropolis* MA7213 was first dissolved in 1 mL of ionic liquid in a glass HPLC vial and made up to 4 mL in a sterile 250 mL Erlenmeyer flask. To initiate the bioconversion, 16 mL of fermentation broth was added into the ionic liquid. The culture was returned to the same incubation conditions (pH ~6.5, 30°C, 200 rpm) for the duration of the bioconversion.

The extent of conversion was monitored by regular sample collection. In the case of water miscible, single phase forming ionic liquids, the samples were centrifuged at 13,000 rpm for 5 minutes in a refrigerated benchtop centrifuge and the supernatant was taken and stored at 4°C prior to analysis. For water immiscible ionic liquids that formed two-phase systems, the samples were similarly centrifuged but the cell free ionic liquid phase was taken and diluted in acetonitrile prior to analysis. All bioconversions were performed in triplicate.

In the case of 1-indanone oxidation by *E. coli* TOP10 pQR239 the culture was grown for a further 4 hours after induction by L(+)-arabinose (total 7 hours cultivation). The cells were then pelleted by centrifugation at 4,000 rpm for 3 minutes. The media was removed without disturbing the pellet and the cells were resuspended in 20 mL 100 mM potassium phosphate dibasic buffer (Cruz *et al*, 2004). The substrate was pre-dissolved in 5 mL of appropriate co-solvent such that the final concentration was 0.5 gL^{-1}. Bioconversion was initiated by the addition of the substrate/co-solvent mixture to the cells and the flask was returned to the previous incubation conditions. Conversion was monitored by regular sample collection and samples were prepared for reverse phase HPLC analysis (Section 2.8.1) as described above.

In the case of miniature bioreactor scale bioconversions with whole cells, *T. capitatum* MY1890 was cultured for 36 hours as described in Section 2.6. The cell suspension was subsequently centrifuged at 4000 rpm in a Sorvall centrifuge for 5 minutes to pellet the cells. The media was removed and the cells were subsequently resuspended in 50 mL KH_2PO_4 buffer. 16 mL of the resuspended cells were pipetted into a Multimax™ miniature bioreactor which contained 4 mL of [Bmim][PF_6] with

6-Br-β-tetralone pre-dissolved to give an overall concentration of 1 gL^{-1}. In parallel, a shake flask bioconversion was carried out using the same cell suspension as described above. Conversion in both cases was monitored by reverse phase HPLC analysis (Section 2.8.1).

2.7.2 Isolated enzyme bioconversions

The initial screening of bioconversions involving a range of co-solvents was carried out at 10 mL reaction volume in jacketed, cone shaped pH stats (Mettler Toledo). 500 mg substrate was added directly to the vessel and the appropriate co-solvent added. They were then mixed to dissolve the substrate at 30°C. Mixing of each vessel was by a magnetic stirrer bar (d = 10 mm) at high speed. After 1 hour, 7 mL of buffer was added with glucose (for co-factor recycling) pre-dissolved to give a final overall concentration of 1.5 molar equivalents of substrate. NAD was dissolved in 1 mL of buffer to give a final NAD concentration of 1 gL^{-1}, and was added to the vessel. The pH was then corrected to 6.8 ± 0.1. Once the pH was appropriately adjusted, 1 mL further of buffer was added containing ADH RE and GDH 103 to final concentrations of 5 gL^{-1} and 1 gL^{-1} respectively. Samples of 50 μL were regularly taken to determine the extent of conversion by dissolving them into 950 μL acetonitrile in a HPLC vial. These were then further diluted 1/10 into acetonitrile and assayed by reverse phase HPLC (Section 2.8.1).

Subsequent studies of bioconversion kinetics were performed in miniature Multimax™ reactors (Mettler Toledo, Columbia, MS, USA) of 30 mL reaction volume fitted with overhead pitched blade impellers (d_i = 24mm). The vessel was

heated by a circulating water jacket and temperature and pH were continuously monitored. Reactions were performed as in the pH stat experiments with adjustments made for the additional volume.

In both configurations pH in the aqueous phase was controlled by the addition of 2.0 N sodium hydroxide.

2.8 Analytical methods

2.8.1 Reverse phase HPLC analyses

A Dionex DX 500 HPLC system (Surrey, UK) equipped with a Zorbax RX-C8 column (4.6mm x 25cm) (Mac-Mod Analytical, Chadds Ford, PA, USA) was employed for quantification of the substrate 6-Br-β-tetralone and the product 6-Br-β-tetralol. Separation was by isocratic elution using a mobile phase of acetonitrile (50% v/v) and acidified water (0.1% v/v 3M phosphoric acid) at a flow rate of 1.0 mL min^{-1}. Detection was by UV detector at 220 nm. The maximum coefficient of variance of this assay was 3.8%.

A rapid assay was also developed where an Agilent series 1100 HPLC system equipped with a Zorbax SB-C18 column (4.6 mm x 50 mm) (Mac-Mod Analytical, Chadds Ford, PA, USA) was employed for the separation of 6-Br-β-tetralone and 6-Br-β-tetralol by isocratic elution with a mobile phase of 50% v/v acetonitrile and acidified water (0.1% v/v phosphoric acid) at a flow rate of 1.0 mL min^{-1}. Detection

was by UV detector at 220 nm. The assay runtime was 4 minutes with substrate eluting at 2.2 minutes and product at 1.6 minutes.

In the case of 4'-2,2,2-trifluoroacetophenone an Agilent series 1100 HPLC system equipped with a Zorbax SB-C18 column (4.6 mm x 50 mm) (Mac-Mod Analytical, Chadds Ford, PA, USA) was employed for the separation of the substrates and products. Separation was by isocratic elution by a mobile phase of 60% v/v acetonitrile and acidified water (0.1% v/v phosphoric acid) at a flowrate of 1.75 mL min^{-1}. Detection was by UV detection at 215nm and the assay runtime was 5 minutes with substrate eluting at 1.3 minutes and product at 2.6 minutes. The maximum coefficient of variance observed was 4.3%.

For the separation of 1-indanone and 3,4-dihydrocoumarin a Dionex DX 500 HPLC system (Surrey, UK) equipped with a Zorbax SB-C18 column (4.6mm x 50mm) (Mac-Mod Analytical, Chadds Ford, PA, USA) was employed. Separation was by isocratic elution using a mobile phase of acetonitrile (30% v/v) and acidified water (0.1% v/v 3M phosphoric acid) at a flow rate of 0.75 mL min^{-1}. Detection was by UV detector at 220 nm and run time 10 minutes with substrate eluting at 4.1 minutes and product at 5.9 minutes.

2.8.2 Chiral analyses

An Agilent series 1100 HPLC system equipped with a Chiralcel OD-H column (Daicel Chemical Industries Limited, USA) was employed for the separation of the two enantiomers of 6-Br-β-tetralol using a mobile phase of hexane with 2% v/v IPA

modifier isocratically at a flowrate of 1.75ml min^{-1} and detection at 210nm. The runtime was 20 minutes with substrate eluting at 9.8 min, the (*S*) product enantiomer at 12.2 min and the (*R*) enantiomer at 16.1 min.

A Supercritical Fluid Chromatography system equipped with a Chiralcel AD-H column was employed for the separation of the two enantiomers of 4'-Br-2,2,2-trifluoroacetophenyl alcohol. A mobile phase of supercritical CO_2 modified with 4% v/v methanol for four minutes was used, which was then increased by linear gradient to 40% v/v methanol at a rate of 2% min^{-1} with a three minute hold at 40% v/v methanol (total run time 25 minutes). The assay was run at 35°C and 200 bar pressure with a mobile phase flowrate of 1.5 mL min^{-1}. Detection was by UV detection at 215 nm with the (*S*) enantiomer eluting at 10.5 and (*R*) at 12.2 minutes.

2.8.3 Biomass quantification

Biomass determinations were made off-line for *T. capitatum* MY1890 by the collection of a 5 mL sample of culture, 1 mL of which was filtered by vacuum filtration through a pre-weighed and dried 0.2 μm filter paper. The cake was washed twice using equal volumes of RO water and held under vacuum until the filter cake became dry. The filter paper and cake were then transferred to a Mettler Toledo AG (Greifensee, Switzerland) HG53 Halogen Moisture analyzer and dried at 110°C until the weight became constant. The difference between the initial dried filter paper weight and the final weight was taken as the dry cell weight in the sample. The measurements were taken in triplicate (maximum coefficient of variance observed

was 14.7%). In the case of *R. erythropolis* MA7213 and *E. coli* TOP10 pQR239 growth was followed by monitoring of absorbance at 600 nm in a spectrophotometer.

2.8.4 Enzyme activity assays

For determination of ADH RE residual activity in the bioconversion studies two solutions were made up: solution A containing 2.5 μL p-Cl acetophenone in 7 mL 200 mM potassium phosphate dibasic buffer (pH~7.2) and solution B containing 24.5 mg NADH in 7 mL buffer. 175 μL and 35 μL respectively were added into a single well of a 96 well micro titre plate. 10 μL of sample was added to a separate well and the assay was initiated by adding 190 μL of the assay solution mixture to the sample. Absorbance at 340 nm was recorded every 20 seconds over 2 minutes and plotted. The slope of this line compared to a standard of known enzyme concentration gave the effective enzyme concentration of the sample. For GDH 103 a single reaction mixture was required containing 24.5 mg NAD and 71.9 mg of glucose in 7 mL buffer. The samples were analysed as for ADH RE. (Maximum coefficient of variance observed was 11.9 %)

2.8.5 Viable cell counts

In the case of *T. capitatum* MY1890 samples were taken at regular intervals once the cell culture had been contacted with the ionic liquid. A 0.1 mL sample was taken and was diluted through a serial dilution cascade and then 0.1 mL of diluted sample was spread on Sabouraud 2% glucose agar plates. The plates were incubated at 28°C for

48 hours. The number of visible colonies (> 50) on the lowest dilution plate was recorded as the number of colony forming units.

In the case of *R. erythropolis* MA7213 and *E. coli* TOP10 pQR239 samples were taken at regular intervals once the cell culture had been contacted with the ionic liquid. Each 0.1 mL sample was taken and was diluted through a serial dilution cascade. These diluted samples were then taken and 0.1 mL was spread on LB agar plates. The plates were incubated at 37°C for 24 hours. The number of visible colonies (> 50) on the lowest dilution plate was recorded as the number of colony forming units.

2.8.6 Enzyme half-life studies

Enzyme (1 mg) was dissolved in 900 μL of 100 mM potassium phosphate dibasic buffer (pH 6.8) in a HPLC vial and 100 μL of appropriate co-solvent was added. These were shaken at 1400 rpm and 30°C on a thermomixer (Eppendorf) and samples periodically taken and assayed for enzyme activity as described in Section 2.8.4. Half life was determined as the time taken for the activity to reach half that of the original activity.

2.8.7 Light microscopy

A 100 μL sample of bioconversion medium was taken and diluted into 900 μL of potassium diphosphate buffer (50mM). 10 μL of dilute sample was transferred onto the surface of a glass slide and was covered by a glass cover slip. The slide was

inspected by a Leica MRA2 (Leica Microsystems, Buckinghamshire, UK) upright microscope and images taken by a CCD camera (Cohu Inc., Berkshire, UK).

2.8.8 Flow cytometry

Flow cytometric analyses were conducted using a Beckman Coulter Epics XL analyser (Beckman Coulter, Buckinghamshore, UK). Stock solutions of dyes were prepared as follows: PI was prepared at a concentration of 1 gL^{-1} in Dulbecco's buffered saline (DBS, pH ~7), BOX was prepared to the same concentration in DMSO, and $DiOc_6(3)$ was prepared to a concentration of 1 mgL^{-1} in DMSO. All solutions were filtered using a 0.2μm syringe filter prior to use.

Culture samples were diluted with DBS such that samples were analysed at a rate of ~500 events s^{-1}. The appropriate stains were added such that the working concentration of PI, BOX and $DiOc_6(3)$ were 5 μgmL^{-1}, 10 μgmL^{-1} and 5 $ngmL^{-1}$ respectively. Samples were allowed to incubate for 5 minutes in the presence of the dyes prior to analysis. The total number of cells analysed per sample was 50,000 resulting in a standard error of <0.33%. Any spectral overlap between dyes used simultaneously was eliminated through the colour compensation feature of the control software. Three replicates were assayed for each sample.

2.8.9 Determining ionic liquid density

A weighing boat was pre weighed and 1 mL of ionic liquid was added using a sterilin 5 mL pipette to minimise loss of sample due to adherence to the pipette interior. The

weighing boat and ionic liquid were reweighed and the weight of ionic liquid was used to calculate ionic liquid density. Each ionic liquid was tested in triplicate. The maximum coefficient of variance observed was 9.4 %.

2.9 Racemic standard synthesis

A chemical reduction of 6-Br-β-tetralone and 4'-Br-2,2,2-trifluoroacetophenone was necessary to generate product standards for reverse phase HPLC analysis (Section 2.8.1). Each substrate was dissolved in ethanol to a concentration of 10 gL^{-1} and 0.5 molar equivalents of sodium borohydride was added in a fume hood. The mixture was stirred using a magnetic flea for 30 minutes and then concentrated HCl was added drop wise to remove excess hydrogen. The product was then extracted into toluene by a half volume extraction and the alcohol isolated by rotary vacuum evaporation of the toluene. Product purity was confirmed by reverse phase HPLC (Section 2.8.1).

2.10 Product isolation

2.10.1 (S)-6-Br-β-tetralol

A simple isolation procedure was feasible whereby the product in the immiscible ionic liquid can be separated from the aqueous phase by centrifugation followed by three half-volume extractions with toluene from the ionic liquid (~60% w/w average extraction efficiency). The product was then isolated from the toluene by vacuum evaporation of the solvent

2.10.2 (R)-4'-Br-2,2,2-trifluoroacetophenyl alcohol

In order to isolate the product, this mixture was taken, centrifuged, and the water layer removed. The product and remaining substrate was then extracted from the ionic liquid layer by 4 washes with diethyl ether until >99% w/w of product was recovered (as determined by reverse phase HPLC). The isolated product from the original reaction with [BMP][NTf2] was recovered by rotary vacuum evaporation of the diethyl ether leaving a viscous oil identified as (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol by reverse phase HPLC (Section 2.8.1) and SFC analysis (Section 2.8.3).

2.11 Lewis cell operation

The kinetics of the mass transfer of 6-Br-β-tetralone from the ionic liquid [BMP][NTf2] and toluene to an aqueous buffer phase (50mM KH_2PO_4) were examined within the Lewis cell. The Lewis cell was designed and constructed as described by Lye and Stuckey (2001). The experiments were performed such that the Reynolds number (Re) in each phase was equal to ensure comparable hydrodynamics on either side of the interface. Reynolds number is defined as:

$$Re = \frac{\rho N d_i^2}{\mu}$$

where N is the impeller speed (s^{-1}) determined using a Lachtron tachometer model DT-2236, d_i is the impeller diameter (m), ρ is the liquid density (kgm^{-3}) and μ is the viscosity (PaS). The Lewis cell was operated at 30°C ± 0.1 in all experiments and this

was maintained by a temperature controlled water bath circulating water through the vessel jacket. Equal volumes of the two phases had first been equilibrated, in the absence of substrate, to ensure that this was the only transferring species. After separation, substrate was added to the ionic liquid or toluene phase such that the overall concentration for the vessel would be 50 gL^{-1}. The lower phase was then funnelled to the bottom of the vessel to avoid wetting the upper half of the cell. The upper phase was subsequently added such that the bottom layer was not disturbed. Both phases were brought to temperature and samples of each phase taken before the impellers were switched on at their pre-set speeds. Each experiment lasted for 4 hours and samples of 50 μl were taken each hour for analysis as described in Section 2.8.1.

3 Whole cell mediated bioreductions of 6-Br-β-tetralone*

3.1 Introduction and aims

The chiral alcohol (*S*)-6-Br-β-tetralol is a key precursor in the synthesis of the anti arrythmia drug candidate MK-0499 (Tschaen *et al*, 1995). Chemical reduction of the ketone 6-Br-β-tetralone to its corresponding (*S*) alcohol yields only a 20% enantiomeric excess of the desired enantiomer. Screening of microbial libraries identified the yeast *Trichosporon capitatum* MY1890 (Reddy *et al*, 1996) and gram positive bacterium *Rhodococcus erythropolis* MA7213 (Stahl *et al*, 2000) that mediated production of the required alcohol at >99% ee. These systems use organic solvents as co-solvents for the bioconversion to dissolve sufficient quantities of substrate to deliver an efficient process.

The aim of this initial chapter will be to explore the feasibility of performing whole cell bioreductions in the presence of ionic liquids. To date, there have only been two preliminary reports on whole cell bioreductions with ionic liquids and these have focussed solely on baker's yeast (Howarth *et al*, 2001; Pfruender *et al*, 2004), as described in Section 1.7. These studies involved a study of the feasibility of performing whole cell bioreductions in the presence of a single ionic liquid ([Bmim][PF_6]). Broadly, this chapter will investigate the impact of a

* The results presented in this chapter have been published as: Hussain W, Pollard DJ, Lye GJ. (2007) **The bioreduction of a β-tetralone to its corresponding alcohol by the yeast *Trichosporon capitatum* MY1890 and the bacterium *Rhodococcus erythropolis* MA7213 in a range of ionic liquids.** *Biocatal Biotransform* **25**: 443-452.

range of ionic liquids on not only the bioreduction, but also the effects of the ionic liquid on the whole cell biocatalyst. Specific objectives of this chapter will be to:

- Examine bioreductions of 6-Br-β-tetralone mediated by both yeast and bacterial cells known to express the same tetralone reductase activity.
- Screen a range of ionic liquids, with varying chemical and physical properties, as co-solvents with regard to substrate solubility and bioconversion kinetics.
- Attempt to correlate bioconversion performance to ionic liquid physico-chemical properties and their impact on cell viability.

3.2 Biocatalyst cultivation and standard bioconversion kinetics

In order to confirm the data previously reported for *T. capitatum* MY1890 (Reddy *et al*, 1996) growth curves were first obtained showing the high cell density (~25-30 gL^{-1}) it is possible to achieve with this organism. A typical growth curve, obtained from a culture grown in a 250 mL shake flask is shown in Figure 3.1 and is comparable to the previously published growth profile for this organism given the different scales of culture (Reddy *et al*, 1996). Growth on glycerol containing media is significantly greater than that observed with glucose, indicating the preference of the organism for less complex carbon sources. For the shake flask culture a final biomass concentration of 26 gL^{-1} was achieved. Bioconversion was initiated after 36 hours growth while the cells were in exponential growth phase and metabolic activity is at a maximum.

The ability to reduce 6-Br-β-tetralone is not exclusive to *T. capitatum* MY1890 but to a number of organisms with a broad range of physiologies and properties (Stahl *et al*, 2000). Among these is the gram positive bacterium *R. erythropolis* MA7213 and the growth curve for this strain is also shown in Figure 3.1. The total biomass yield is significantly less than for *T. capitatum* MY1890, reaching a maximum dry cell weight of around 4.5 gL^{-1}. The exponential phase of growth for *R. erythropolis* MA7213 appears to last from around 10 to 24 hours. The ideal time to initiate bioconversion is seen to be around 24 hours, when the cell density is close to it's maximum and the cells are still actively growing.

Figure 3.2(a) shows a standard bioconversion time-course for *T. capitatum* MY1890 which was initiated after 36 hours growth by the addition of 10% v/v ethanol containing 6-Br-β-tetralone. The bioconversion begins instantaneously as reflected by the rapid initial rate of product formation of 70 $mg(product)g^{-1}(cell)hr^{-1}$. The substrate uptake rate is initially even higher at 157 $mg(substrate)g^{-1}(cell)hr^{-1}$ as reflected by the deficit in the solute mass balance during the initial 3 hours of reaction. This is attributed to the association of substrate with the cell surface or its rapid initial uptake and accumulation within the cell.

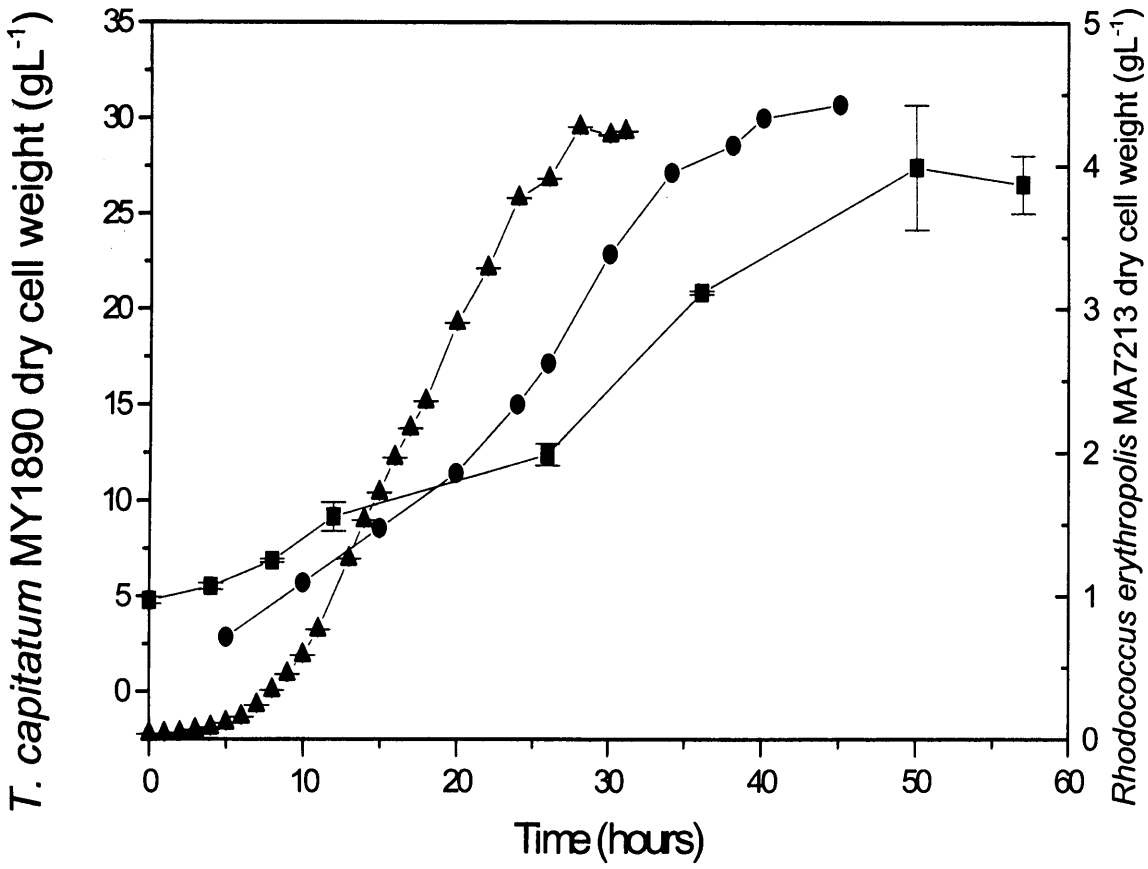


Figure 3.1 Comparison of growth of (■) *T. capitatum* MY1890 on 30gL^{-1} glycerol based medium in a 250 mL shake flask, maintained at 28°C on a shaking incubator (220 rpm), with surface aeration, (▲) *R. erythropolis* MA7213 grown on 30 gL^{-1} TSB in a 250 mL shake flask, maintained at 30°C on a shaking incubator (200 rpm), with surface aeration and (●) *T. capitatum* MY1890 on 60 gL^{-1} glycerol, in a 23L fermenter maintained at 28°C, agitation at 220 rpm by impeller, with aeration of 6L of air per minute and DOT maintained at 30% of initial saturation by control of aeration and impeller speed (Reddy *et al*, 1996). Shake flask cultivation was performed as described in Section 2.6 and biomass concentration was determined as described in Section 2.8.3. Error bars indicate one standard deviation about the mean.

By the end of the reaction (after 5 hours) the mass balance closes to 100% as expected and complete conversion of the substrate is achieved.

In comparison Figure 3.2(b) shows the standard bioconversion time-course for *R. erythropolis* MA7213, again in the presence of 10% v/v ethanol. As in the case of *T. capitatum* MY1890, the rate of substrate uptake is significantly higher than the rate of product formation giving a similar deficit in the mass balance. However the product formation rate remains slow and the final conversion is only 14% w/w. This is due in part to the six-fold decrease in cell density in the case of the *R. erythropolis* MA7213 bioconversion compared to *T. capitatum* MY1890. It was found that lysing the cells after the reaction phase was completed released the unconverted substrate from within the cell completing the mass balance and indicating the substrate was not converted by other enzyme activities within the cell.

In both cases, chiral analysis (performed as described in Section 2.8.2) of the bioconversion product showed >99% enantiomeric excess in favour of the desired (*S*) enantiomer.

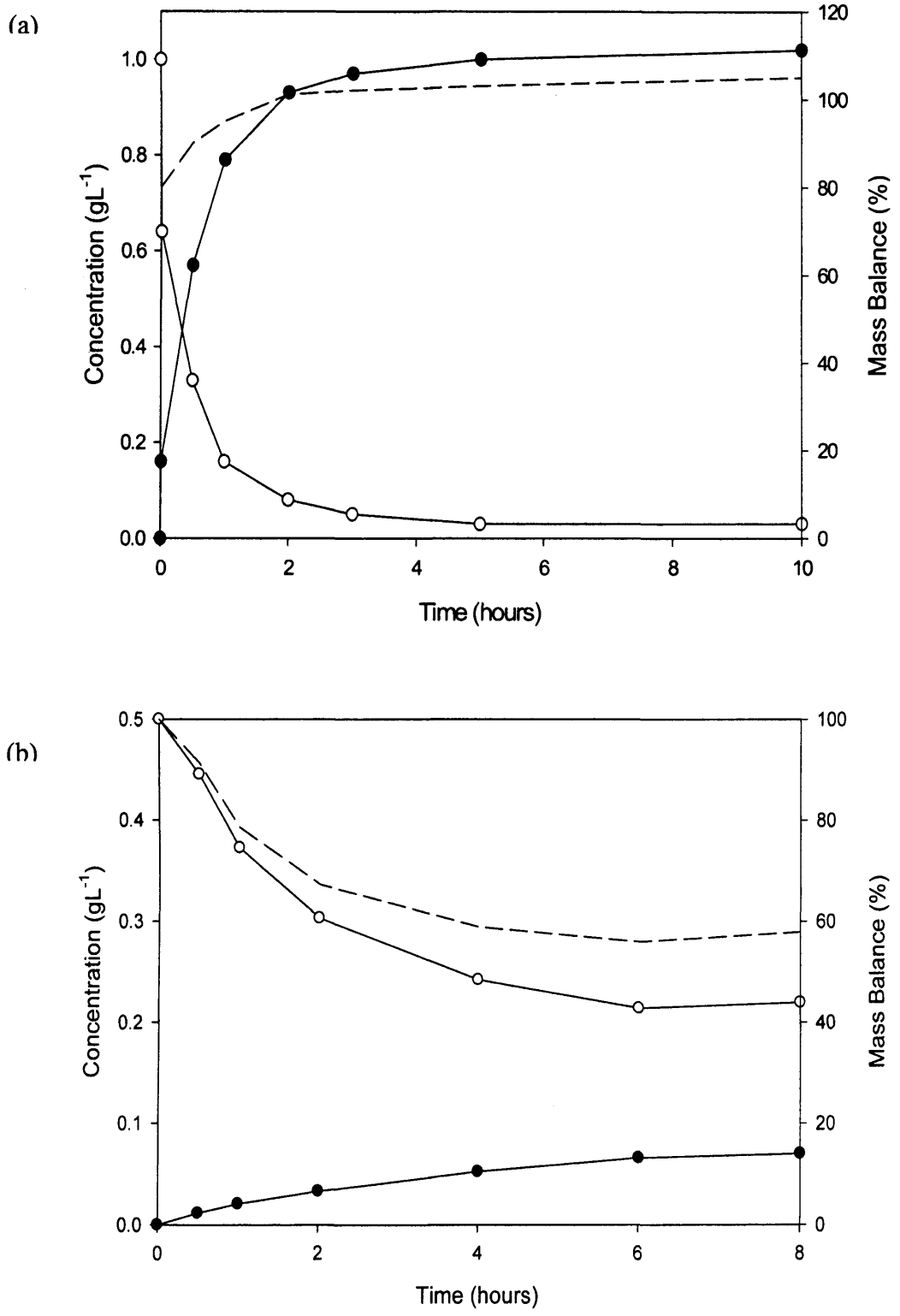


Figure 3.2 Time course of typical bioreductions of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by (a) the yeast *T. capitatum* MY1890 and (b) the gram positive bacterium *R. erythropolis* MA7213: (o) 6-Br-β-tetralone ; (●) 6-Br- β-tetralol ; (-) mass balance. Experimental conditions were as described in Sections 2.6 for microbial cultivation and 2.7.1 for the bioconversion.

3.3 Ionic liquid screening

3.3.1 Physical properties

Prior to studying the activity of the biocatalysts in ionic liquids it was necessary to investigate some of the physical properties of the ionic liquids that could have an effect on the processing of these liquids at scale. Table 3.1 shows a number of the key physical properties for the limited range of seven ionic liquids used in this study. The first property is water miscibility, which affects whether the system runs as a single phase or a two-phase system. It is clear that miscibility is dependent upon the anion/ cation combination. This is exemplified by the ionic liquids [Bmim][BF_4], which is completely miscible with water, while [Bmim][PF_6] is very hydrophobic and mixes with water only to a limited extent (Sheldon, 2001). The choice of monophasic or biphasic system will potentially affect the rate of reaction, ease of product recovery and downstream processing.

The density and viscosity of the ionic liquids will affect the ways in which the ionic liquids can be mixed in the reaction vessel, and the ease of pumping the liquids in industrial scale systems. Generally the published values for the viscosities of the pure ionic liquids are many orders of magnitude greater than the viscosity of water, the maximum viscosity being that of [CABHEM][$MeSO_4$] at 3 Pas, compared to 9 x 10^{-2} Pas. However, during this work it was noted that the viscosity of the ionic liquids decreased dramatically upon the introduction of even a small amount of water (~1% v/v) to the system. With added water the viscosities are greatly reduced becoming much closer to that of water and as such

do not pose problems for large scale operation. The densities of the pure ionic liquids range from 825-1370 kgm^{-3} which will not pose any significant processing issues other than those with higher densities than water will form the lower phase in the case of water immiscible ionic liquids.

Literature data concerning ionic liquids has shown they are effective solvents dissolving many complex molecules to high titres (Roberts and Lye, 2002). A potential advantage of ionic liquids for biocatalysis is the ability to dissolve significantly more substrate in the environment of the catalyst than conventional solvents. However, as shown in Table 3.1, this benefit could not be realised in this work as the 6-Br-β-tetralone substrate proved difficult to dissolve in any of the ionic liquids tested at concentrations higher than that achieved with the standard ethanol co-solvent.

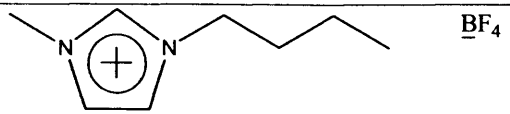
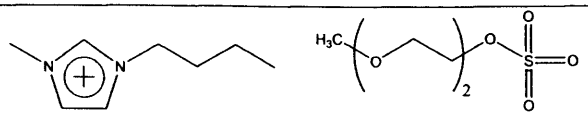
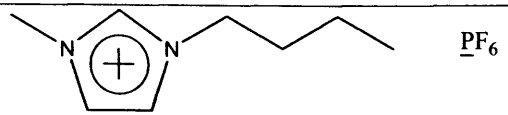
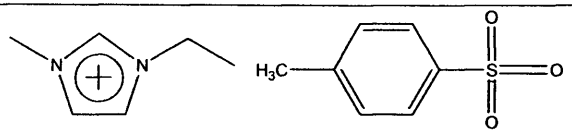
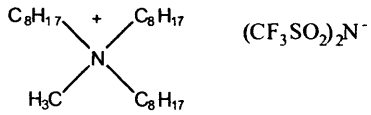
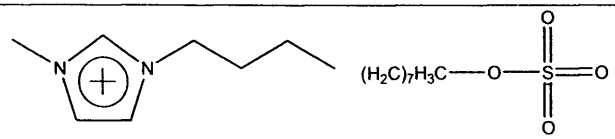
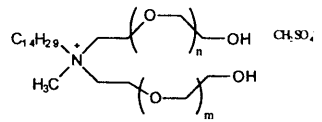
Name	Ionic Liquid Structure	Abbreviation	Water miscibility	Density kg m^{-3}	Viscosity PaS	Tetralone solubility gL^{-1}
Media/ Ethanol	N/A	N/A	N/A	1000	0.0001	10.0
1-Butyl-3-methyl-imidazolium tetrafluoroborate		[Bmim][BF_4]	Miscible	1210	0.145	2.1
1-Butyl-3-methyl-imidazolium-ethylenglycolmonomethyl-ethersulfate		[Bmim][$MDEGSO_4$]	Miscible	1060	0.340	3.6
1-Butyl-3-methyl-imidazolium hexafluorophosphate		[Bmim][PF_6]	Immiscible	1370	0.385	4.33
1-Ethyl-3-methyl-imidazolium tosylate		[Emim][TOS]	Miscible	930	n/a	6.35
Methyl-trioctyl ammonium bis(trifluoromethlylsulfonyl)imide		[Oc_3MeN][NTf2]	Immiscible	925	n/a	7.20
1-Butyl-3-methyl-imidazolium octylsulfate		[Bmim][$OcSO_4$]	Miscible	940	0.874	7.9
PEG-5 Cocomonium methosulfate		[CABHEM][$MeSO_4$]	Miscible	825	3.000	4.70
Toluene	N/A	N/A	Immiscible	850	0.00065	>50

Table 3.1 Ionic liquid property table. Tetralone solubility was determined by saturating the ionic liquid with β-tetralone and subsequent HPLC analysis of a diluted sample. Viscosities of the ionic liquid are literature values for pure ionic liquids (www.solventinnovation.com). Density was determined independently as described in Section 2.8.9. n/a indicates data not available.

3.3.2 Cell viability upon exposure to ionic liquids

Cell viability, as measured by viable plate counts (Section 2.8.5), may be taken as an initial quantitative measure of the response of cellular systems on exposure to ionic liquids. The rate of decrease in *T. capitatum* MY1890 viable cell counts upon exposure to various ionic liquids is shown in Figure 3.3(a) and is summarised in Table 3.2 (log N/N_0 data). Contact with the ionic liquid at a concentration of 50% v/v has a significant effect on the cells for the first 2-4 hours, with as many as 5 log reductions in viable cell counts. The effects of all the ionic liquids fall within the boundaries formed by media and the two commonly used organic co-solvents ethanol and toluene (Figure 3.3(a)). These results indicate that although the ionic liquids are to some degree toxic to the cells, they can be less so than typical organic solvents used in industrial bioconversions.

A comparison of the *T. capitatum* MY1890 viability profiles (Figure 3.3(a)) to bioconversion kinetics profiles, such as that for ethanol (Figure 3.2(a)), shows that despite the rapid loss of cell viability the reaction still proceeds to completion. Plate counts are indicative of intact, polarised and metabolically active cells that are also capable of cell division. This comparison, then, shows that while there is a large reduction in cell viability nonviable cells can still regenerate cofactor and complete the reduction over the time scales studied here as has been found for bioconversions with conventional solvents. With the measured reductions (>2 log reductions)

though it would appear to be unfeasible or uneconomic to recover and recycle the biocatalyst.

Figure 3.3(b) shows the changes in cell viability in the case of *R. erythropolis* MA7213 and are summarised in Table 3.3 (log N/N_0 data). In general, the gram positive bacterium can be seen to be much more tolerant to the presence of ionic liquids in comparison to the yeast strain. The ionic liquids tested appear to fall into two categories. A number of ionic liquids have very little effect on viable cell number over the 8 hours of measurement; single phase forming ionic liquids such as [Bmim][BF_4] and [Emim][TOS] caused log reductions of less than 0.1, along with the two-phase forming ionic liquids [Bmim][PF_6] and [Oc_3MeN][NTf2]. In contrast, ionic liquids such as [Bmim][$OcSO_4$] caused a much greater decrease in viability in a similar way to toluene. The greatly increased stability of *Rhodococcus* species in certain ionic liquids compared to organic solvents is in agreement with earlier findings from this laboratory (Cull *et al*, 2000). In this case however there appears to be a closer correlation between measured biocompatibility and bioconversion kinetics: those ionic liquids that appeared detrimental to the viability of the cells were also poorer co-solvents for the bioconversion.

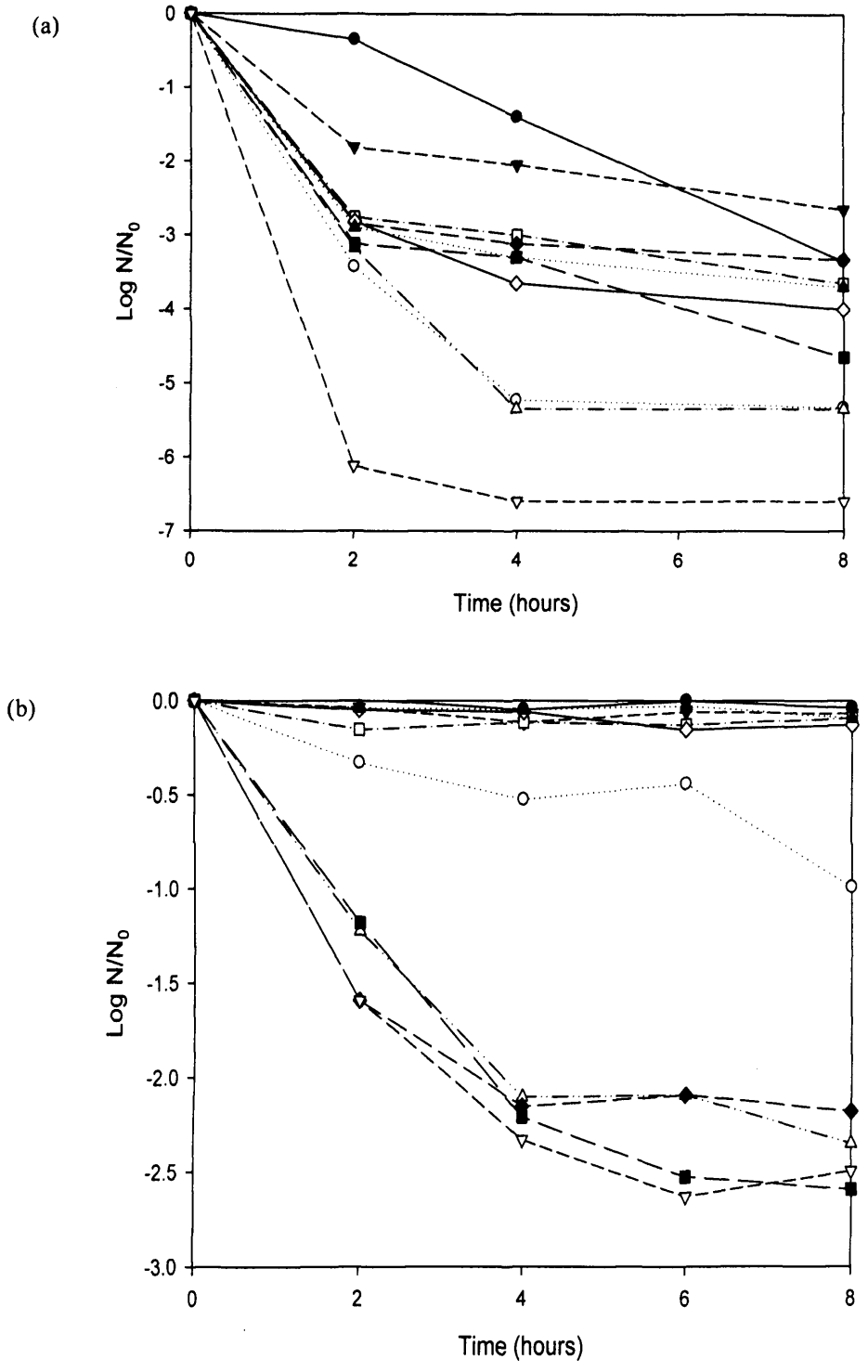


Figure 3.3 Viability of (a) *T. capitatum* MY1890 and (b) *R. erythropolis* MA7213 in various ionic liquids. A shake flask was cultured at 28°C for 36 hours in the case of *T. capitatum* and for 24 hours at 30°C for *R. erythropolis* in a shaking incubator. 5mL of the culture was then taken and contacted with 5mL of ionic liquid (50% v/v) for 8 hours. Periodically samples were taken and serial diluted in phosphate buffer, and the dilutions spread on agar plates. The number of colonies present were counted and normalised against a control of 100% media (Section 2.8.5). (●) media control (○) 10% v/v ethanol (▼) [Bmim][BF_4] (Δ) [CABHEM][$MeSO_4$] (■) [Bmim][$MDEGSO_4$] (□) [Oc_3MeN][NTf2] (♦) [Bmim][$OcSO_4$] (◊) [Bmim][PF_6] (▲) [Emim][TOS] (▽) 10% v/v toluene.

3.4 Bioconversion kinetics in ionic liquids

3.4.1 T. capitatum *MY1890 bioconversions*

Bioconversions catalysed by *T. capitatum* MY1890 were carried out using all of the ionic liquids shown in Table 3.1 employing a volume fraction of ionic liquid of 20% v/v in all cases. Examples of typical reaction profiles are shown in Figure 3.4 (the complete set is given in Appendix B) and the results for all ionic liquids are summarised in Table 3.2. It can be seen that the highest reaction rate is observed with the ionic liquid [Emim][TOS] where the overall rate of reaction is comparable to that observed for the standard bioconversion conditions, and the reaction approaches completion within 4 hours (Figure 3.4(a)). The initial decrease in the solute mass balance is again associated with rapid uptake of substrate into the cells but this is subsequently converted to product which partitions out of the cells restoring the mass balance.

Considering the rest of the hydrophilic ionic liquids, in the case of [Bmim][BF_4] the reaction was observed to proceed steadily in the presence of the ionic liquid, but only to a final conversion of less than 35% w/w. This was surprising since the cell viability data (Table 3.2) showed this ionic liquid to be the least harmful to the cells.

Medium	Number of Phases	Log(N/N_0)	Initial Rate ($mg(prod)g^{-1}(cell)\ hr^{-1}$)	Conversion (% w/w)
Standard	1	-3.35	69.8	100
[Emim][TOS]	1	-3.70	50.9	100
[Bmim][BF_4]	1	-2.65	2.2	35
[Bmim][$OcSO_4$]	1	-3.33	0.7	10
[Bmim][$MDEGSO_4$]	1	-4.65	0.5	11
[CABHEM][$MeSO_4$]	1	<-5.35	3.3	30
[Bmim][PF_6]	2	-4.00	3.1	40
[Oc_3MeN][NTf2]	2	-3.65	4.2	60

Table 3.2 Comparison of ionic liquids for the reduction of 6-Br-β-tetralone by *T. capitatum* MY1890. Cell viability is shown as the $\log_{10}$ of the ratio of viable cells in the ionic liquid after 8 hours to the viable cells in media and is based on Figure 3.3(a). All bioconversions were carried out at 20% v/v ionic liquid or 10% v/v ethanol in the case of the 'standard' medium as described in Section 2.7.1. Initial specific rate was calculated on the basis of product yield in the first hour. Final conversion is shown after 8 hours.

The observed reaction rate of 2.2 $mg(product)g^{-1}(cell)hr^{-1}$ reflects the poor conversion, and may be attributed to the association of the ionic liquid with the cell membrane preventing effective transfer of substrate into the cell. [Bmim][$OcSO_4$] is another water miscible ionic liquid, which performed most poorly of all the ionic liquids tested. In 8 hours, less than 10% w/w conversion was observed (Figure 3.4(b)). The very low initial rate of 0.73 $mg(product)g^{-1}(cells)hr^{-1}$ suggests that the ionic liquid caused extensive damage to the cells, as indicated by the $\log N/N_0$ in Table 3.2, or that the viscous nature of this ionic liquid (Table 3.1) limited the mass transfer of substrate into the cell.

[Bmim][$MDEGSO_4$] also gave a poor conversion of 11% w/w, but this is almost certainly attributed to toxicity of the ionic liquid to the biocatalyst, since a large proportion of the substrate was taken up by the cells in the first hour (Appendix B). This indicates that mass transfer of substrate into the cell, is unlikely to be a limiting factor and failure to convert is likely due to cell death. The final water miscible ionic liquid is [CABHEM][$MeSO_4$] which was the most viscous of the ionic liquids tested. However, once contacted with the aqueous phase at 20% v/v, the dilution effect dramatically reduced the viscosity making it easier to handle. After an initial four hour lag phase the reaction proceeded at an initial rate of 3.3 mg(product)g^{-1}(cell)hr^{-1} and reached a maximum of 30% w/w conversion. A further point of interest with this ionic liquid was that conversion proceeded with a colour change from a deep green to dark brown as the reaction progressed. No alteration in the quality of the substrate and product was observed based upon the retention times and shapes of the HPLC peaks.

Considering the two hydrophobic ionic liquids, [Oc_3MeN][NTf2] and [Bmim][PF_6], these performed much better on average than the majority of hydrophilic ionic liquids in terms of retained cell viability and conversion yield (Table 3.2). This is attributed to the bulk of the ionic liquid being physically separate from the cells present in the aqueous phase, and so having less direct influence upon the biocatalyst. Orbital shaking of the flasks containing the reaction mixture proved effective at mixing the two phases; when the shaking was stopped the two phases

visibly separated after less than a minute. The ionic liquid [Bmim][PF_6], which has been commonly used in chemical catalysis (Sheldon, 2001) also proved effective for the bioconversion studied here. It gave a product yield of 40% w/w, at a constant rate of 3.1 mg(product)g^{-1}(cell)hr^{-1}, indicating the limiting factor is most likely not the viability of the biocatalyst but the mass transfer rate of the substrate into the aqueous phase. The other hydrophobic ionic liquid [Oc_3MeN][NTf2] yielded almost 60% w/w conversion at an initial rate of 4.2 mg(product)g^{-1}(cell)hr^{-1} (Figure 3.4(c)). The rate of product formation again remained approximately constant over the full 8 hours of the reaction period. The solute mass balance remained close to 100% throughout the bioconversion suggesting substrate mass transfer from the ionic liquid was probably rate limiting.

It was found that the chirality (determined as described in Section 2.8.2) of the product formed in the presence of 20% v/v [Emim][TOS], the best of the ionic liquids investigated, was not significantly affected compared to the chirality obtained in the presence of 10% v/v ethanol (both >99% ee in favour of the desired (*S*) enantiomer.

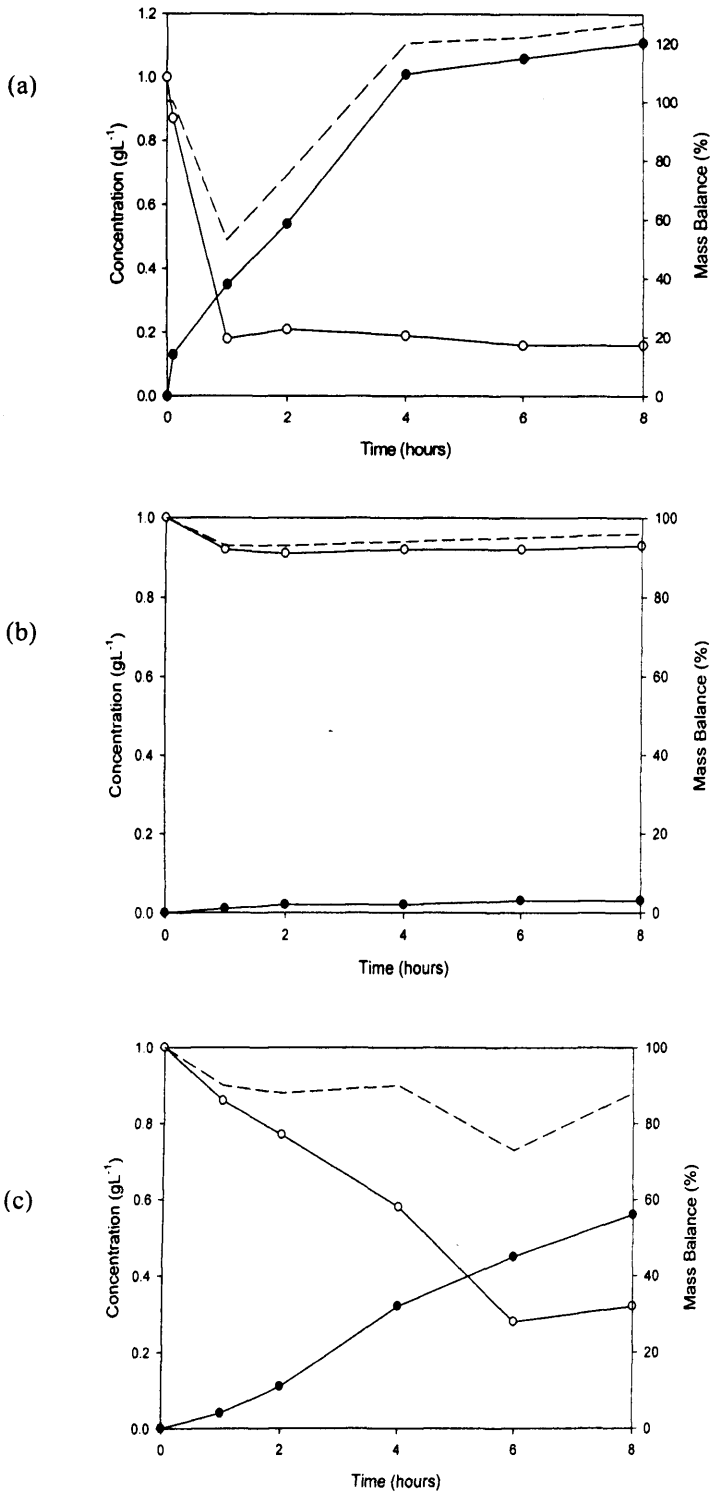


Figure 3.4 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the yeast *T. capitatum* MY1890 in representative ionic liquids (a) [Emim][TOS]; (b) [Bmim][$OcSO_4$]; (c) [Oc_3MeN][NTf2]: (○) 6-Br-β-tetralone; (●) 6-Br- β-tetralol; (-) mass balance. Experimental conditions: 250 mL erlenmeyer flask containing 50 mL of glycerol medium was inoculated with 1mL of cell stock on 10% v/v glycerol and aerobically incubated on an orbital shaker at 28°C and 200rpm. After 36hr bioconversion was initiated by the addition of 16 mL cell culture to 4 mL ionic liquid containing 0.020g 6-Br-β-tetralone. The culture was returned to the same incubation conditions and the extent of conversion was monitored by HPLC analysis as described in Section 2.8.1.

3.4.2 R. erythropolis *MA7213 bioconversion*

Bioconversions catalysed by *R. erythropolis* MA7213 were also carried out using all of the ionic liquids shown in Table 3.1 employing a volume fraction of 20% v/v in all cases. The cell viability, initial rate of reaction and conversion data are summarised in Table 3.3.

Medium	Number Phases	Log(N/N_0)	Initial Rate (mg(prod)g^{-1}(cell) hr^{-1})	Conversion (% w/w)
Standard	1	-0.99	4.7	14
[Emim][TOS]	1	-0.09	18.7	28
[Bmim][BF_4]	1	-0.07	4.6	11
[Bmim][$OcSO_4$]	1	-2.18	9.4	9
[Bmim][$MDEGSO_4$]	1	-2.59	1.2	1
[CABHEM][$MeSO_4$]	1	-2.35	2.3	3
[Bmim][PF_6]	2	-0.13	3.6	7
[Oc_3MeN][NTf2]	2	-0.09	15.0	17

Table 3.3 Comparison of ionic liquids for the reduction of 6-Br-β-tetralone by *R. erythropolis* MA7213. Cell viability is shown as the $\log_{10}$ of the ratio of viable cells in the ionic liquid after 8 hours to the viable cells in media at the start. All bioconversions were carried out at 20% v/v ionic liquid or 10% v/v ethanol in the case of the 'standard' medium. Initial specific rate was calculated on the basis of product yield in the first hour. Final conversion is shown after 8 hours.

Of all the ionic liquids tested the highest initial rate of 18.8 mg(product)g^{-1}(cell)hr^{-1} was observed with the hydrophilic ionic liquid [Emim][TOS] with an overall conversion of 28% w/w (Figure 3.5). The cells also showed one of the smallest

decreases in cell viability upon exposure to this ionic liquid. The performance of the rest of the hydrophilic ionic liquids varied between 1-11% w/w overall conversion with the performance showing some correlation to the viable cell count data. Of the hydrophobic ionic liquids [Oc_3MeN][NTf2] gave the best performance with an initial rate of 15 mg(product)g^{-1}(cell)hr^{-1} close to that observed with [Emim][TOS], but a lower conversion of 17%. Substrate mass transfer from the ionic liquid phase was again likely to be rate limiting.

Although the conversion of 6-Br-β-tetralone by *R. erythropolis* MA7213 is significantly less than observed in the presence of *T. capitatum* MY1890, the initial rate of product formation in the presence of 20% v/v [Emim][TOS] is actually about 4 fold greater than under standard conditions using 10% v/v ethanol (Table 3.3). The conversion yield is also doubled suggesting a positive advantage of ionic liquids for this particular bioconversion. The cell viability data $\log(N/N_0)$ indicates there is an approximately 10 fold decrease of cell viability in the presence of ethanol compared to [Emim][TOS]. Microscopic observation of the *R. erythropolis* MA7213 cells during bioconversion showed that in the presence of ethanol the cells formed large aggregates while in the presence of [Emim][TOS] the cells stayed largely dispersed as in fermentation media (Figure 3.6(b)). This aggregation of cells will almost certainly result in a mass transfer limitation of substrate into the aggregates as reflected by the lower measured initial rates of reaction. In the case of *T. capitatum* MY1890 (Figure 3.6(a)) no aggregation was observed following bioconversions in the presence of either ethanol or [Emim][TOS].

In summary, comparable reactions between the ionic liquid [Emim][TOS] and ethanol were demonstrated with both the gram negative and gram positive cultures, *T. capitatum* MY1890 (Figure 3.2(a), 3.4(a)) and *R. erythropolis* MA7213 (Figure 3.2(b), 3.5). The effect of employing the ionic liquid as a co-solvent upon the increase in initial rate of reaction was significantly more marked in the case of *R. erythropolis* MA7213 than in *T. capitatum* MY1890.

3.5 Summary

From the results reported here, it is clear that ionic liquids are feasible media for whole cell bioreduction involving co-factors. Comparable or better initial rates and final conversions have been observed for both *T. capitatum* MY1890 (a yeast) and *R. erythropolis* MA7213 (a gram positive bacterium) in the presence of ionic liquids compared to a standard ethanol co-solvent (Tables 3.2 and 3.3 respectively). Ionic liquids offer advantages that can be used to improve processes in ways that were not possible when using organic solvents, such as the opportunity to use much greater proportions of ionic liquid to aqueous media, thereby increasing the overall concentration of substrate in the environment of the cell. This is evidenced by the fact that much greater proportions of ionic liquid (up to 50% v/v) have been tested with the cells and they have remained not only capable of performing the desired reaction (Figures 3.4(a) and 3.5), but they also remain viable and able to divide as shown by their ability to form colonies on agar plates (Figure 3.3).

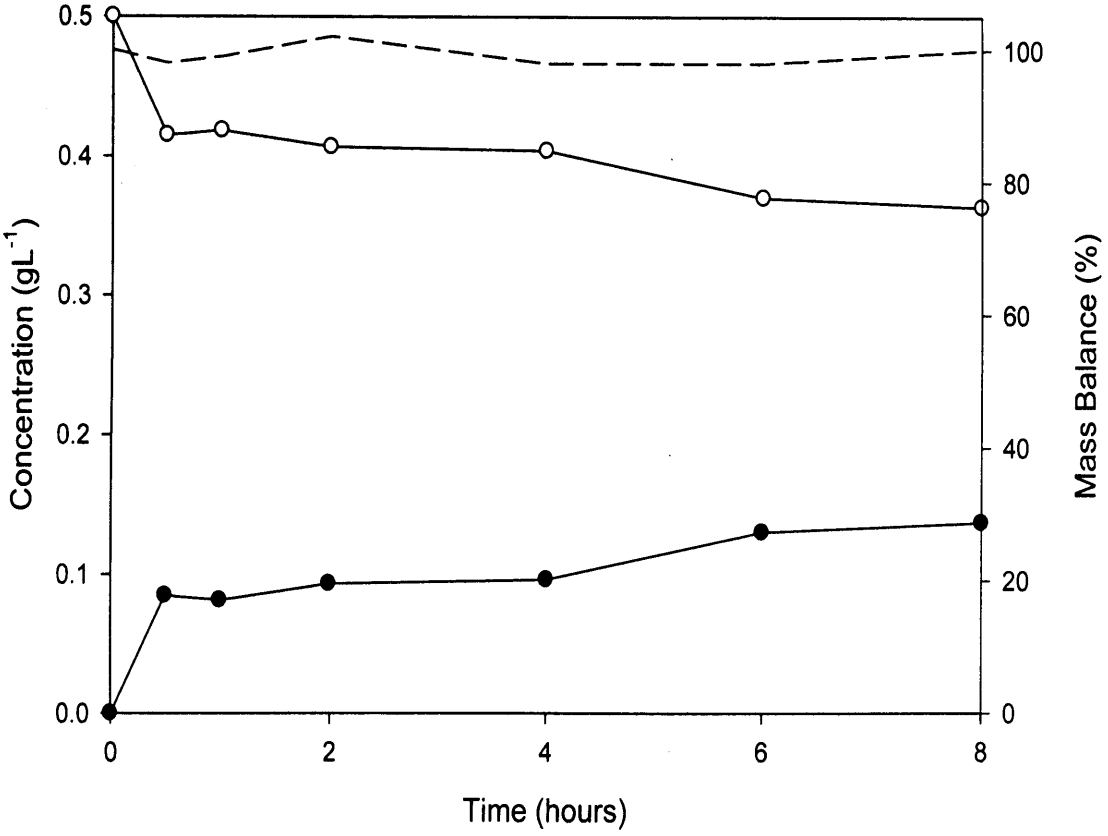


Figure 3.5 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by *R. erythropolis* MA7213 in the presence of [Emim][TOS]: (○) 6-Br-β-tetralone gL^{-1}; (●) 6-Br- β-tetralol gL^{-1}; (-) mass balance. Experimental conditions: 250 mL Erlenmeyer flask containing 50mL of Tryptic Soy Broth ($30gL^{-1}$) was inoculated with 1mL of cell stock on 10% v/v glycerol and was aerobically incubated on an orbital shaker at 28°C and 200 rpm. After 24 hrs biotransformation was initiated by the addition of 16 mL cell culture to 4ml ionic liquid containing 0.020g 6-Br-β-tetralone. The culture was returned to the same incubation conditions and the extent of conversion was monitored by regular sample collection.

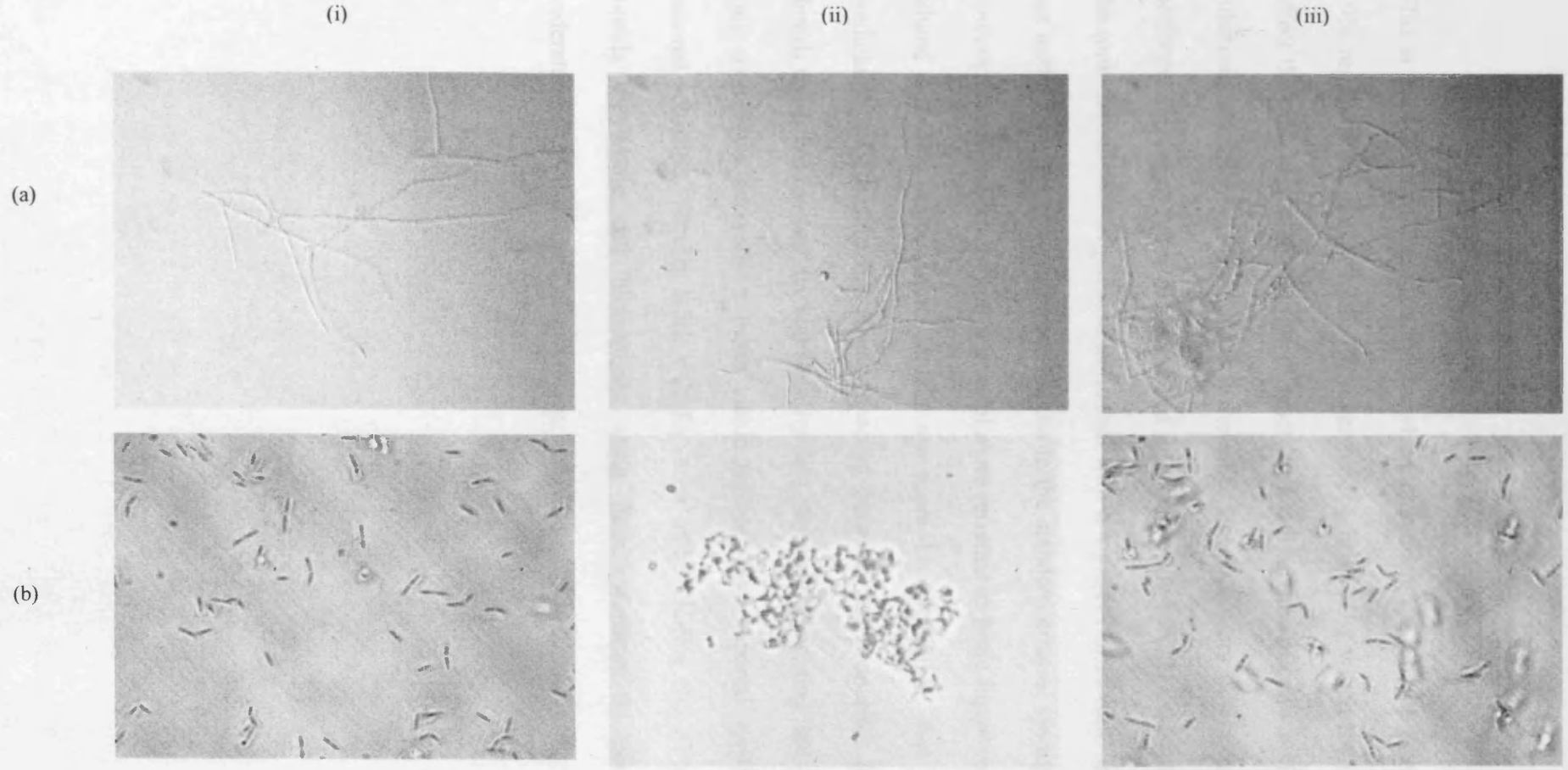


Figure 3.6 Light microscope images of (a) *T. capitatum* MY1890 (40X magnification) and (b) *R. erythropolis* MA7213 (100X magnification) after (i) cultivation in growth medium (36 hr and 21 hr respectively) and during bioconversions in the presence of (ii) 10% v/v ethanol or (iii) 20% v/v [Emim][TOS] (30minutes after bioconversion is initiated). Bioconversions were performed as described in Section 2.7.1 and images were obtained as described in Section 2.8.7.

This is in contrast to organic solvents where increasing the volume fraction beyond 10% results in rapid cell death. However the mechanism by which the ionic liquids affect the cells and alters the rate of reaction is still unknown: cell viability data and substrate solubility do not appear to be sufficient indicators of biotransformation performance, for example in the case of *R. erythropolis* MA7213 the toxic effect of the ionic liquid [Bmim][BF_4] is minimum with a $\log_{10}(N/N_0)$ of -0.07 after 8 hours but initial rate and conversion are similar to the standard ethanol system. It is clear however that some strains are in general more resistant to ionic liquid exposure than others, and that some ionic liquids are more biocompatible than others. No predictable relationship has been observed between the properties of the ionic liquids used, and the activity and stability data observed, indicating that screening of ionic liquids is currently a more viable method for industrial application than rational selection. Further work will focus on understanding the effects of ionic liquids on whole cell biocatalysts using flow cytometry to gain a better understanding of the interaction between ionic liquids and bacterial cells.

4 Quantifying the impact of ionic liquids on whole cell biocatalyst stability

4.1 Introduction and aims

4.1.1 Introduction

In the previous chapter the ability of whole cells of both *T. capitatum* MY1890 and *R. erythropolis* MA7213 was demonstrated for the reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol in the presence of ionic liquids. The results suggested that bioconversion efficiency was, at least in part, related to the viability of the whole cell biocatalysts (Section 3.4.1) with ionic liquids demonstrating improved biocompatibility compared to a number of common organic solvents (Section 3.3.2). Viability was determined from simple plate counts after exposure to the ionic liquids. The disadvantages of plate counts are that they are only a gross measure of a cells potential to divide and can take up to 24 hours before viability results can be obtained for some organisms.

4.1.2 Flow cytometry

As described in Section 1.8, flow cytometry analysis of a cell population can reveal, at a cell by cell level, the physiological condition of a cell population such as cell viability, cell density, function or ion density. The parameters investigated for a particular cell line or application can be tuned by employing

appropriate dyes that, upon excitation by a laser, emit at different wavelengths to allow analysis of multiple parameters per cell in a single run.

Flow cytometry is built on the premise that a cell population can be identified through a characteristic size and "granularity"- determined through forward and side scattering of the laser in the system (Vale-Silva and Buchta, 2006). By "gating" this characteristic area and determining base values of fluorescence for each of the dyes on those events, changes can readily be identified by shifts in these fluorescence intensities.

4.1.3 Aims

Flow cytometry is a powerful analytical technique providing a rapid and detailed insight into the response of individual cells in a population on exposure to non-aqueous solvents. The aim of this chapter is to use flow cytometry to obtain an understanding of the response of various types of biocatalyst on exposure to ionic liquids. Given the filamentous nature of *T. capitatum* MY1890 (Figure 3.6(a)) and the principle of operation of flow cytometry (Figure 1.15) it was not possible to study *T. capitatum* MY1890 using this method. Consequently two bioconversions were chosen for this study. The first is the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the gram positive bacterium *R. erythropolis* MA7213 as described in Section 1.9.1. The second is the Baeyer-Villiger oxidation of 1-indanone to 3,4-dihydrocoumarin by the gram negative bacterium *E. coli* TOP10 pQR239 (Section 1.9.3). The pQR239 plasmid confers *Acetinobacter caloaceticus* cyclohexanone mono-oxygenase (CHMO) activity on

the *E. coli* TOP10 host, as well as ampicillin resistance useful as a selection marker (Doig *et al*, 2001). Literature data has shown the bioconversion shown in Figure 4.1 proceeds with "minimum conversion" in the presence of 10% v/v ethanol (Guttierez *et al*, 2003). The potential for ionic liquids to be used to achieve a greater degree of conversion is investigated here through the use of flow cytometry to identify potentially favourable ionic liquids.

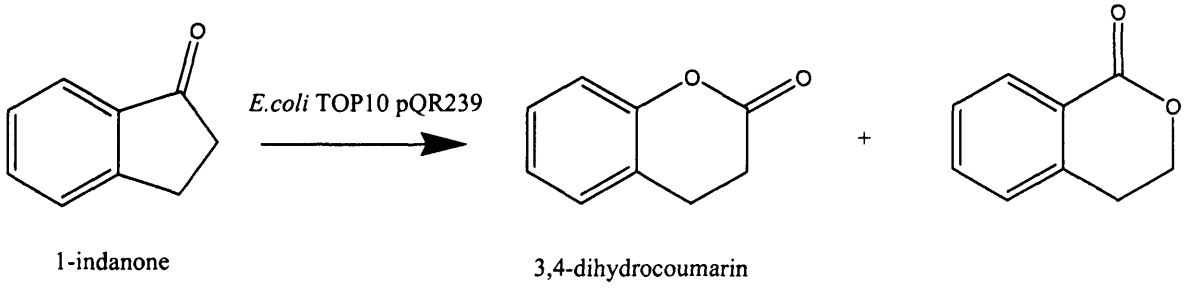


Figure 4.1 The CHMO mediated oxidation of 1-indanone to 3,4-dihydrocoumarin by *E. coli* TOP10 pQR239.

The specific objectives of this chapter are:

- To establish the use of flow cytometry as a potential high throughput method for quantifying the impact of ionic liquids on whole cell biocatalyst stability.
- To attempt to correlate the measured response of *R. erythropolis* MA7213 and *E. coli* TOP10 pQR239 biocatalysts to the structure and properties of a range of ionic liquids.
- To establish if there is any relationship between flow cytometry results, conventional plate counts (Section 3.3.2) and bioconversion performance.

4.2 Flow cytometry analysis and reference conditions

4.2.1 R. erythropolis *MA7213*

The first whole cell biocatalyst selected for investigation using flow cytometry was *R. erythropolis* MA7213 (Section 1.9.1) and suitable dyes were sought for identifying membrane polarization and cell membrane permeabilisation (Section 1.8). Propidium iodide (PI) is a double stranded DNA stain very commonly used for identifying cell membrane permeabilisation (Lehtinen *et al*, 2004; Looser *et al*, 2005; Baatout *et al*, 2006; Berney *et al*, 2007; Saegeman *et al*, 2007). PI cannot pass through an intact cell membrane to stain the DNA inside a cell so only cells not metabolizing or controlling their internal environment will be positively stained (Amanullah *et al*, 2002).

Membrane polarization is achieved through the maintenance of a charge balance across the membrane by potassium/ sodium ion channels. The use of ATP by the cell causes 3 sodium ions to be pumped out of the cell and two potassium ions simultaneously pumped in resulting in a potential difference across the membrane. The maintenance of this potential difference can be taken as an indication of the metabolic activity of the cell, since cells not using ATP or unable to use it would be unable to maintain the charge imbalance (Nebe-von-Caron *et al*, 1998). Membrane permeabilisation is not concomitant of depolarization however so PI will not be able to pass through the membrane and stain the DNA of stressed cells only, and it has been shown that stressed cells can recover (Baatout *et al*, 2006).

The most commonly used membrane depolarisation dye in the flow cytometry literature is bis-oxonol (BOX) (Amanullah *et al*, 2003; Onyeaka *et al*, 2003) and this was the first dye investigated. Due to the gram positive nature of *R. erythropolis* MA7213, and hence the presence of a peptido-glycan cell wall (Koch, 2003), BOX was unable to penetrate the cell wall on depolarization. It was found that only through lengthy heat shock treatment of the cell population, with an associated breakdown of the cell wall, was the BOX able to penetrate the cell. This is clearly unsuitable. Similarly Rhodamine 123 (Rh123), which operates under the same premise as BOX (Amanullah *et al*, 2003), was also unsuitable. The alternative dye, $DiOC_6(3)$, was identified through the flow cytometry literature and was found to be a suitable dye for identifying depolarization of *R. erythropolis* MA7213 (Reis *et al*, 2005). Contrary to the mode of action of BOX and Rh123 this dye stains a polarized cell membrane but is unable to penetrate a depolarized one.

Based on the use of PI and $DiOC_6(3)$ the reference condition plot for *R. erythropolis* MA7213 is shown in Figure 4.2(a). This indicates >99% live, metabolically active cells (quadrant H1) approaching the end of the exponential growth phase after cultivation for 21 hours in Tryptic Soy Broth (30 gL^{-1}). The expected progression of flow cytometry counts upon contact with a detrimental co-solvent is from quadrant H1, representing polarized, live cells, to H3 representing live cells with depolarized membranes and finally to H4 representing cells with permeabilised membranes.

4.2.2 E. coli *TOP10 pQR239*

The parameters to be investigated for this strain are again membrane polarization (as an indirect indicator of metabolism) and membrane permeabilisation as indicated by the PI DNA stain. The bis-oxonol dye ($DiBAC_5$) unsuitable for *R. erythropolis* MA7213 analysis was found to be applicable here for the gram negative *E. coli* cell. Based on the use of PI and BOX the reference plot for cells in their standard medium at the end of the exponential phase of growth is shown in Figure 4.2(b). In this case there are >98% of the cells in quadrant K3, indicating cells negative for both PI and BOX being live and polarized. The expected progression for the flow cytometry counts is from quadrant K3 to K1 indicating live depolarized cells and finally the permeabilised cells will occupy quadrant K2. Quadrant K4, indicating PI positive but BOX negative results will be occupied by DNA in cell debris pockets that appear within the forward and side scatter parameters indicated due to the already very small size of *E. coli* cells.

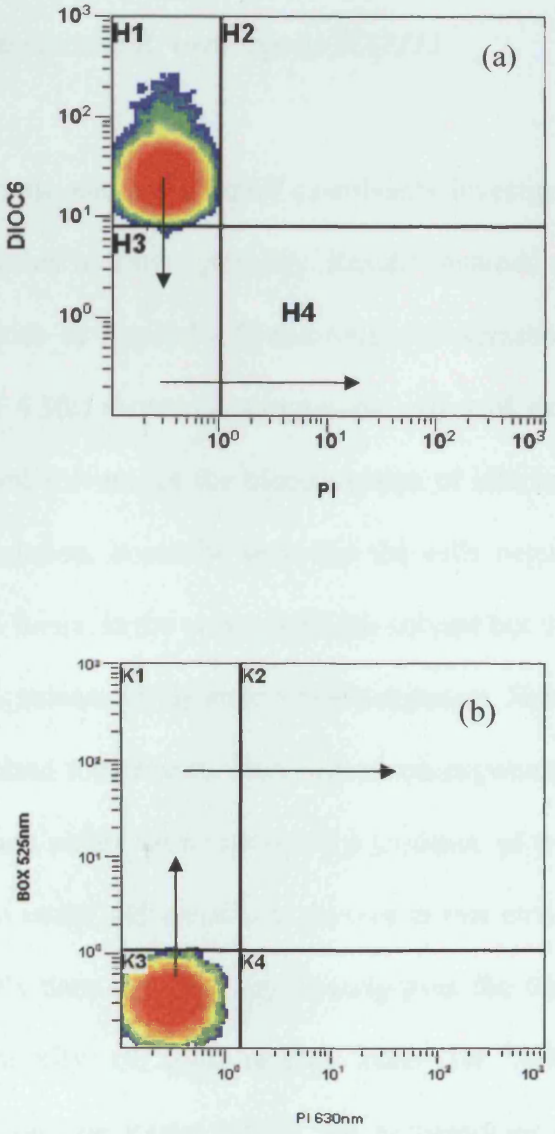


Figure 4.2 Flow cytometry density plots under reference conditions. (a) PI/$DiOC_6$(3) staining of early stationary phase *R. erythropolis* MA7213 cells. Quadrant H1 corresponds to live, polarised cells, H3 corresponds to live cells with depolarised membranes and H4 corresponds to dead cells. (b) PI/BOX staining of stationary phase *E. coli* TOP10 pQR239 cells. Quadrant K3 corresponds to live, polarised cells, K1 corresponds to live cells with depolarised membranes and K2 corresponds to permeabilised cells. Arrows indicate the expected progression of the cell population after exposure to damaging solvents In both cases, a sample of the cells in media was taken and diluted into Dulbecco's Buffered Saline (DBS) prior to flow cytometry analysis as described in Section 2.8.8.

4.3 Effects of co-solvents on *R. erythropolis* MA7213

4.3.1 Flow cytometry analysis of R. erythropolis *MA7213*

The effects of the organic and ionic liquid co-solvents investigated in Section 3.4.2 were analysed further by flow cytometry. Results obtained for all the ionic liquids studied are shown in Appendix C and only representative ones will be described here. Figure 4.3(a) shows the progressive effect of exposure to 10% v/v ethanol, the standard solvent for the bioconversion of interest (Reddy *et al*, 1996) on the cell population. It can be seen that the cells begin to depolarize relatively quickly, <1.5 hours, in the presence of this solvent but that the majority of the cells, about 75%, remain viable after 5 hours exposure. However, only 7% of the cells have polarized membranes upon immediate exposure to the solvent indicating the cells come under stress through the presence of the ethanol. The cell membranes remain intact and are able to survive in this environment as the proportion of dead cells does not vary significantly over the time course. The membrane in the majority of cells remain intact (as indicated by the characteristic forward and side scatter values) and so membrane bound proteins and enzymes are likely to operate successfully.

In contrast Figure 4.3(b) shows the effect of the ionic liquid [Emim][TOS] (20% v/v) on the cell population which appeared to be the most biocompatible ionic liquid of all those tested. Over 99% of the cells are seen to remain viable with polarized and non-permeabilised membranes throughout the time course of the experiment. By comparison Figure 4.3(c) shows that the ionic liquid

[Bmim][$OcSO_4$] is highly toxic to the cells. Greater than 99% of cells were found to have permeabilised membranes within 30 minutes of exposure and 100% were in this state at the times shown in Figure 4.3.

4.3.2 Correlation of flow cytometry data with viable plate counts

Viable cell counting on plates (by determining colony forming number as described in Section 2.8.5) is a much cruder measure of cell population state compared to flow cytometry which picks up more subtle changes in cell physiology. It was hypothesized here that the colony forming unit count determined through plate counting is equivalent to polarized, live cell counts identified through flow cytometry i.e. cells in quadrant H1 of Figure 4.2(a). This is because cells must be live and metabolizing to form new colonies on plates while stressed or permeabilised cells would be unable to go through cell division.

Figure 4.4 shows a parity plot comparing colony forming unit counts (Section 3.3.2) against live polarized cell counts determined here through flow cytometry. In general there is a positive linear correlation between the two measurements confirming the proposed hypothesis. However the correlation coefficient is only 98%. This is due to the nature of the co solvents and their modes of action as the data is skewed to the upper and lower data ranges respectively. Solvents displaying intermediate effects on the cells were relatively rare.

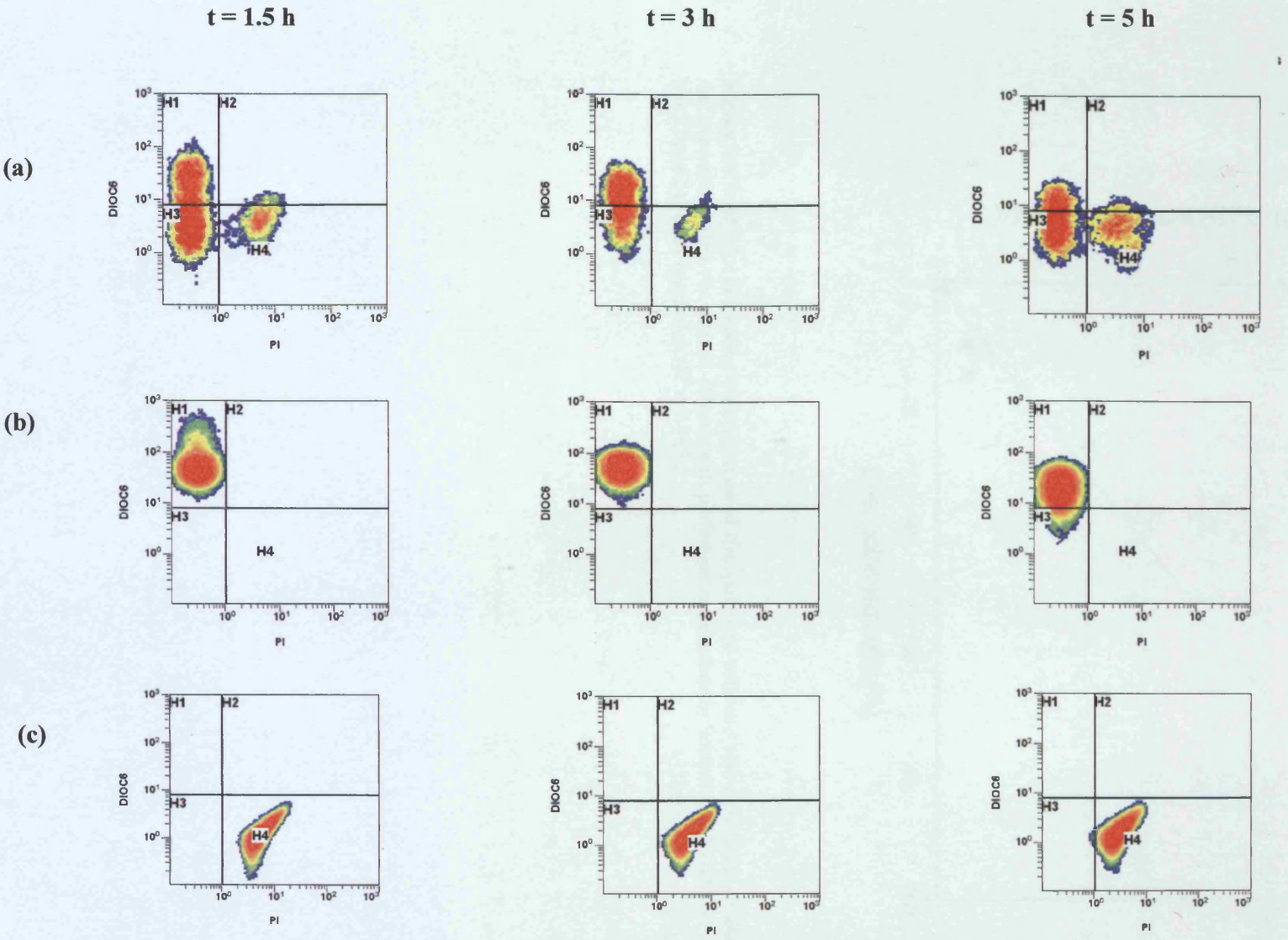


Figure 4.3 Response of *R. erythropolis* MA7213 to exposure to various miscible and immiscible co-solvents. (a) 10% v/v ethanol (b) 20% v/v [Emim][TOS] (c) 20% v/v [CABHEM][$MeSO_4$]. Quadrant H1 corresponds to live, polarised cells, H3 corresponds to live cells with depolarised membranes and H4 corresponds to permeabilised cells. Experiments performed as described in Section 2.8.8.

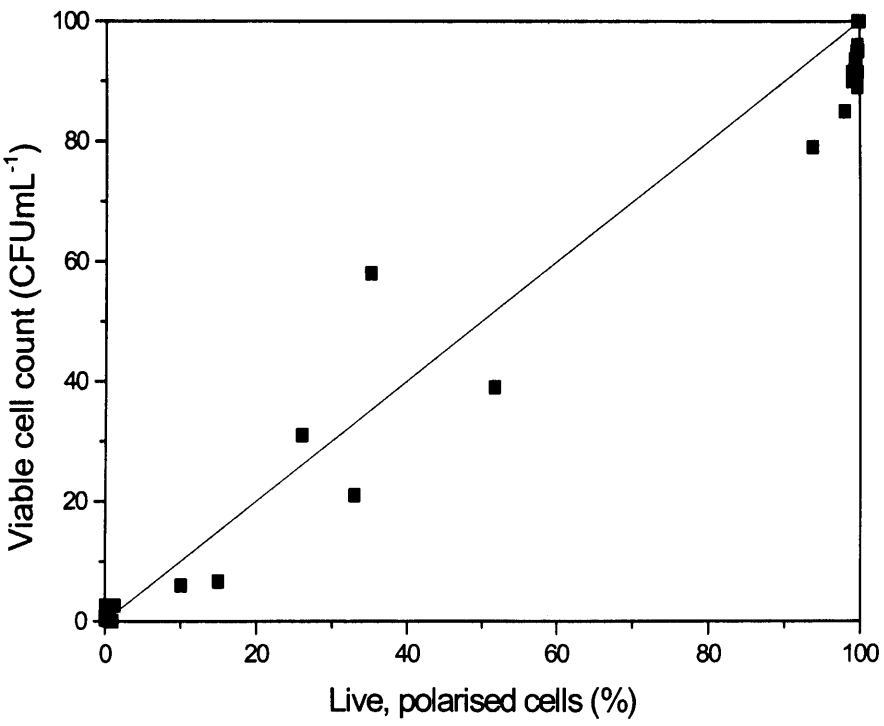


Figure 4.4 Parity plot comparing the percentage of live, polarised cells and viable cell plate counts after cell exposure to 10% v/v ethanol. Experiments performed as described in Sections 2.8.5 and 2.8.8. Solid lined fitted by linear regression ($R^2 = 0.985$).

It should also be remembered that flow cytometry only provides percentages of cells in a certain quadrant from the total number of whole cells it initially identifies due to their characteristic forward and side scatter patterns. Flow cytometry will not take into account grossly deformed cells that lose their characteristic forward and side scatter measures or those cells completely broken down to debris. However such effects will only impact on data in the lower range of measurements. Overall there does appear to be a positive correlation between flow cytometry results and plate count measurements suggesting flow cytometry can be used as a high throughput measure of the whole biocatalyst stability.

4.3.3 Correlation of flow cytometry data and bioconversion efficiency

Figure 4.5 shows the corresponding bioconversion data for *R. erythropolis* MA7213 in the presence of the co-solvents identified in Figure 4.3 along with overall conversion and initial rate data for the reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol. As suggested by the flow cytometry data in Figure 4.3, the cells in the presence of 20% v/v [Emim][TOS] remain viable and are able to convert the ketone at an initial rate of 20 $mg(prod)g^{-1}(cell)hr^{-1}$ to a final conversion of 28% w/w. This is in contrast to 10% v/v ethanol as a co-solvent where only 14% w/w conversion was achieved at a lower initial rate of 5 $mg(prod)g^{-1}(cell)hr^{-1}$. This is in line with the flow

cytometry data in Figure 4.3(a) which shows a much lower proportion of viable cells in quadrant H1 compared to [Emim][TOS]. Finally, the flow cytometry data also successfully predicts the lack of conversion and negligible rate of reaction in the presence of 20% v/v [CABHEM][$MeSO_4$] where the cells were shown to be rapidly permeabilised (Figure 4.3(c)). Overall the results indicate that flow cytometry is a potential tool for rapidly identifying ionic liquids giving high rates of reaction as well as high biocompatibility. This is perhaps to be expected for oxidative reactions where the cells must retain metabolic activity in order to be able to recycle the necessary co-factors.

4.4 Flow cytometry analysis of *E. coli* TOP10 pQR239

4.4.1 Characterisation of 1-indanone bioconversion

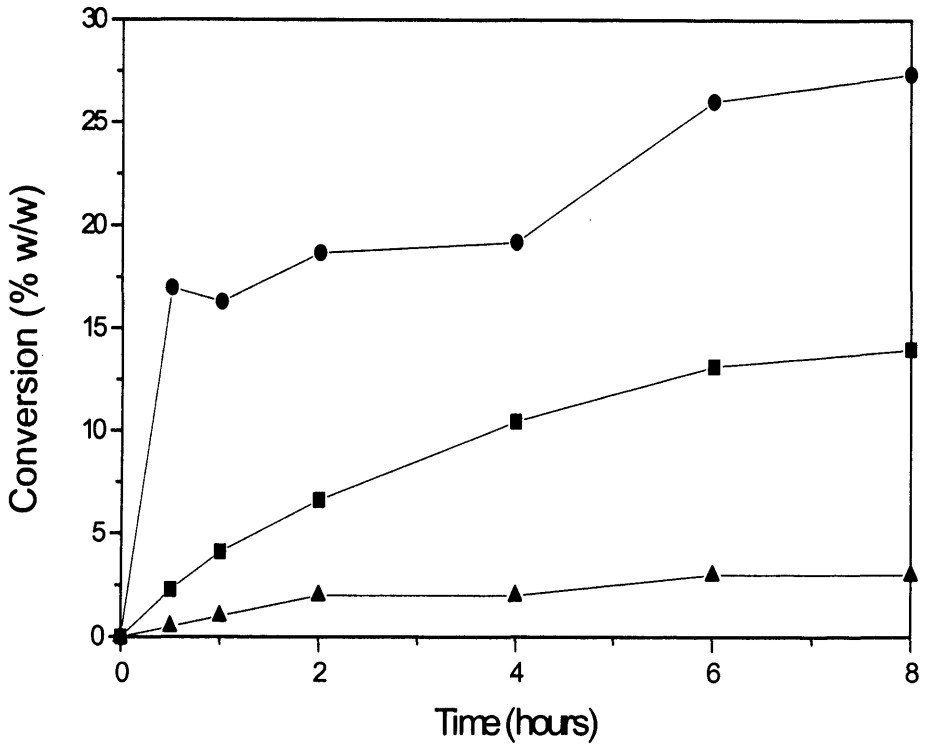
In order to determine the optimum time for induction of CHMO expression and bioconversion initiation, an understanding of the growth kinetics of this whole cell biocatalyst is required. The *E. coli* TOP10 pQR239 was cultured in shake flasks and a growth curve was obtained as shown in Figure 4.6.

Experiments were then carried out to determine the optimum point for induction with L(+)-arabinose by performing 10% v/v ethanol bioconversions with cells induced at different points and determining which yielded the most product. The results of this induction study are shown in Table 4.1, showing the ideal time for induction is between 2 and 4 hours. Consequently the system was subsequently induced at 3 hours and bioconversion initiated after 7 hours cultivation in all subsequent experiments.

Induction Time (hrs)	**Conversion (% w/w)**
0	3
1	9
2	18
3	19
4	19
5	14
6	9
7	1

Table 4.1. Summary of data to the determine optimum time for induction of *E. coli* TOP10 pQR239 culture to maximize conversion of 1-indanone. Induction was carried out as described in Section 2.6. Bioconversion was initiated after 7 hours total culture as described in Section 2.7.1.

(a)



(b)

Co-solvent	Conversion (% w/w)	Initial rate ($g(prod)L^{-1}hr^{-1}$)
Ethanol	14	0.02
[Emim][TOS]	28	0.08
[CABHEM][$MeSO_4$]	3	<0.01

Figure 4.5 Comparison of (a) conversion profiles of 6-Br-β-Tetralone to (*S*)-6-Br-β-Tetralol in the presence of (●) 20% v/v [Emim][TOS] (■) 10% v/v ethanol (▲) 20% v/v [CABHEM][$MeSO_4$] (b) initial rates of reaction and conversion of these reactions. Microbial cultivation was carried out as described in Section 2.6, and bioconversion was subsequently performed as described in Section 2.7.1.

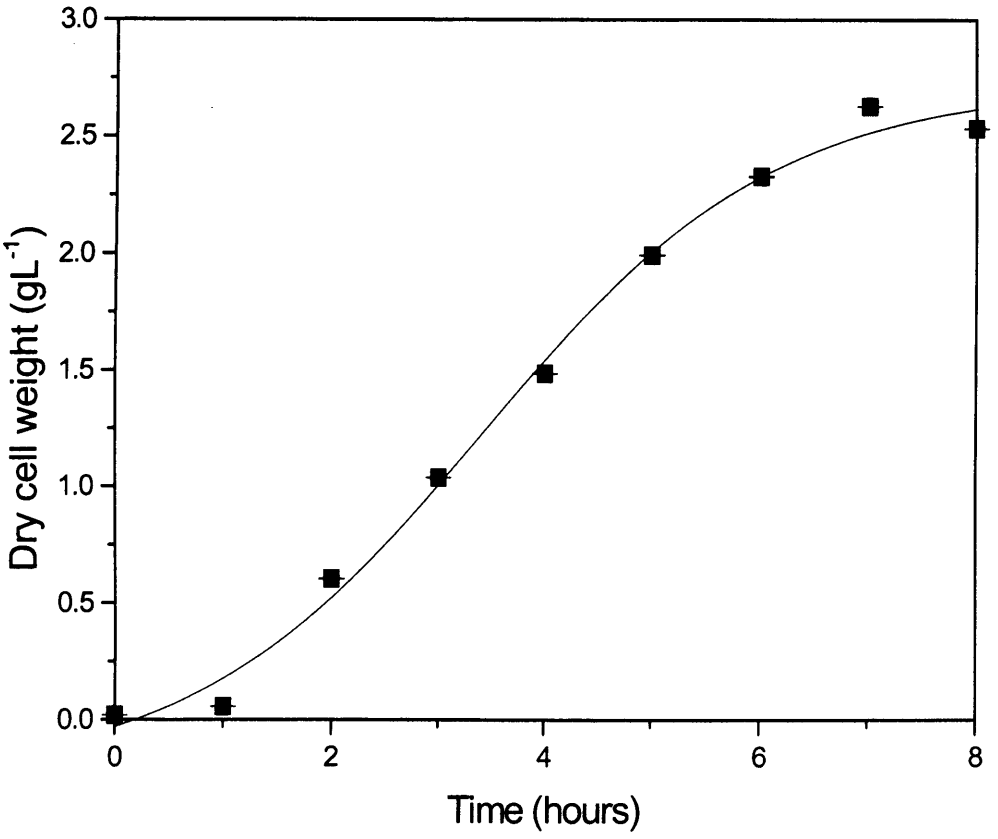


Figure 4.6 Growth profile of *E. coli* TOP10 pQR239 on modified LB media in a 250 mL Erlenmeyer flask over 8 hours at 37°C in a shaking incubator operating at 200 rpm with surface aeration. Microbial cultivation was performed as described in Section 2.6 and biomass analysis as described in Section 2.8.3. Error bars indicate one standard deviation about the mean.

A further parameter of interest is substrate solubility (as described in Section 3.3.1), both in pure co-solvent (100% v/v) and subsequent solubility in the aqueous phase upon initiation of bioconversion. The solubility results are summarised in Table 4.2. As was observed in the case of 6-Br-β-tetralone, solubility of 1-indanone in the ionic liquid is less than in the organic solvent, with ethanol and toluene solubility of indanone both over 700 gL^{-1}. Conversely, the highest solubility with ionic liquids is in AmmoEng™ 102. Of the 2 ionic liquids identified in the flow cytometry analysis of *R. erythropolis* MA7213, 1-indanone solubility is around 350 gL^{-1} in both at 100% v/v, with the hydrophilic [Emim][TOS] conferring 2.2 gL^{-1} on the aqueous phase at 20% v/v and the hydrophobic [Oc_3MeN][NTf2] conferring around 0.3 gL^{-1} on the aqueous phase at 20% v/v ionic liquid.

4.4.2 Flow cytometry analysis of E. coli *TOP10 pQR239*

Figure 4.7 shows the flow cytometry analysis of *E. coli* TOP10 pQR239 in the presence of 10% v/v ethanol, 20% v/v [Emim][TOS] and 20% v/v [CABHEM][$MeSO_4$]. Results obtained for all the ionic liquids studied are shown in Appendix C.

Solvent (% v/v)	Substrate Solubility (gL^{-1})			
	10	**20**	**50**	**100**
EcoEng™ 212	1.37	2.48	8.53	248
[Bmim][PF_6]	0.41	0.43	0.7	313
AmmoEng™ 102	2.86	4.87	12.96	456
[Bmim][$OcSO_4$]	1.91	3.19	7.99	295
AmmoEng™ 120	1.92	3.81	9.37	446
[Oc_3MeN][NTf2]	0.32	0.34	1.72	331
[Emim][TOS]	1.21	2.19	8.75	362
[Bmim][BF_4]	1.40	2.57	5.43	273
[CABHEM][$MeSO_4$]	2.18	2.36	5.25	192
[BMP][BTI]	0.30	0.31	0.32	301

Table 4.2 Summary of 1-indanone solubility at various co-solvent volume fractions. In the case of 10-50% v/v co-solvent the solubility recorded is that in the aqueous buffer phase. In the case of 100% co-solvent the solubility shown is the saturation substrate solubility in the co-solvent Experiments were performed as described in Section 2.3.

In the presence of 10% v/v ethanol (Figure 4.7(a)) around 50% of cells become permeabilised in the first half an hour increasing to over 80% within 2 hours. However, contrary to this behaviour in the presence of 20% v/v [Emim][TOS] (Figure 4.7(b)) approximately 45% of cells remain polarized with intact membranes, with approximately 30% depolarized inside the first half hour, changing to 38% polarized with intact membranes and 60% with permeabilised membranes in 2 hours. In the case of [CABHEM][$MeSO_4$] (Figure 4.7(c)) approximately 90% of cells become permeabilised within the first 10 minutes indicating the much greater toxicity of this ionic liquid to these cells.

Figure 4.8 shows the flow cytometry data obtained for *E.coli* TOP10 pQR239 in the presence of the hydrophobic, two-phase-forming ionic liquid [Oc_3MeN][NTf2]. As expected, the partitioning of the solvent appears to have a protecting effect on the cells as the toxic components of the ionic liquid partition much more slowly into the aqueous phase, and washing of the ionic liquid removed many of the soluble impurities before use (Section 2.4). The bar chart in Figure 4.8(b) shows the intact cell population is maintained at 50-60% throughout the analysis. Also, the gradual depolarization and subsequent death of the cells can be more readily tracked in this "slower" system.

It can be seen that in the case of permeabilised cells multiple "sub-populations" of cells become apparent with different characteristic fluorescences. The presence of these sub-populations is an artifact of the use of cell stocks to produce seed cultures rather than colonies from plates. Although the sub populations are clearly apparent, they do not affect the validity of the results as they are plasmid carrying cell populations with CHMO expression as evidenced by their ampicillin resistance.

Overall in the case of *E. coli* TOP10 pQR239 the flow cytometry data indicates [Emim][TOS] and [Oc_3MeN][NTf2] as single phase forming and two phase forming biocompatible ionic liquids respectively.

4.4.3 E.coli *TOP10 pQR239 bioconversion kinetics*

The bioconversion kinetic data for the oxidation of 1-indanone to 3,4-dihydrocoumarin by *E. coli* TOP10 pQR239 is summarised in Figure 4.9. Table 4.2 summarises the calculated initial rates of reaction and biocatalyst half life as calculated from the flow cytometry data in the presence of a range of co-solvents. Conversion in the presence of ethanol reaches around 20% w/w within five hours at an initial rate of around 0.07 $g(prod)L^{-1}hr^{-1}$.

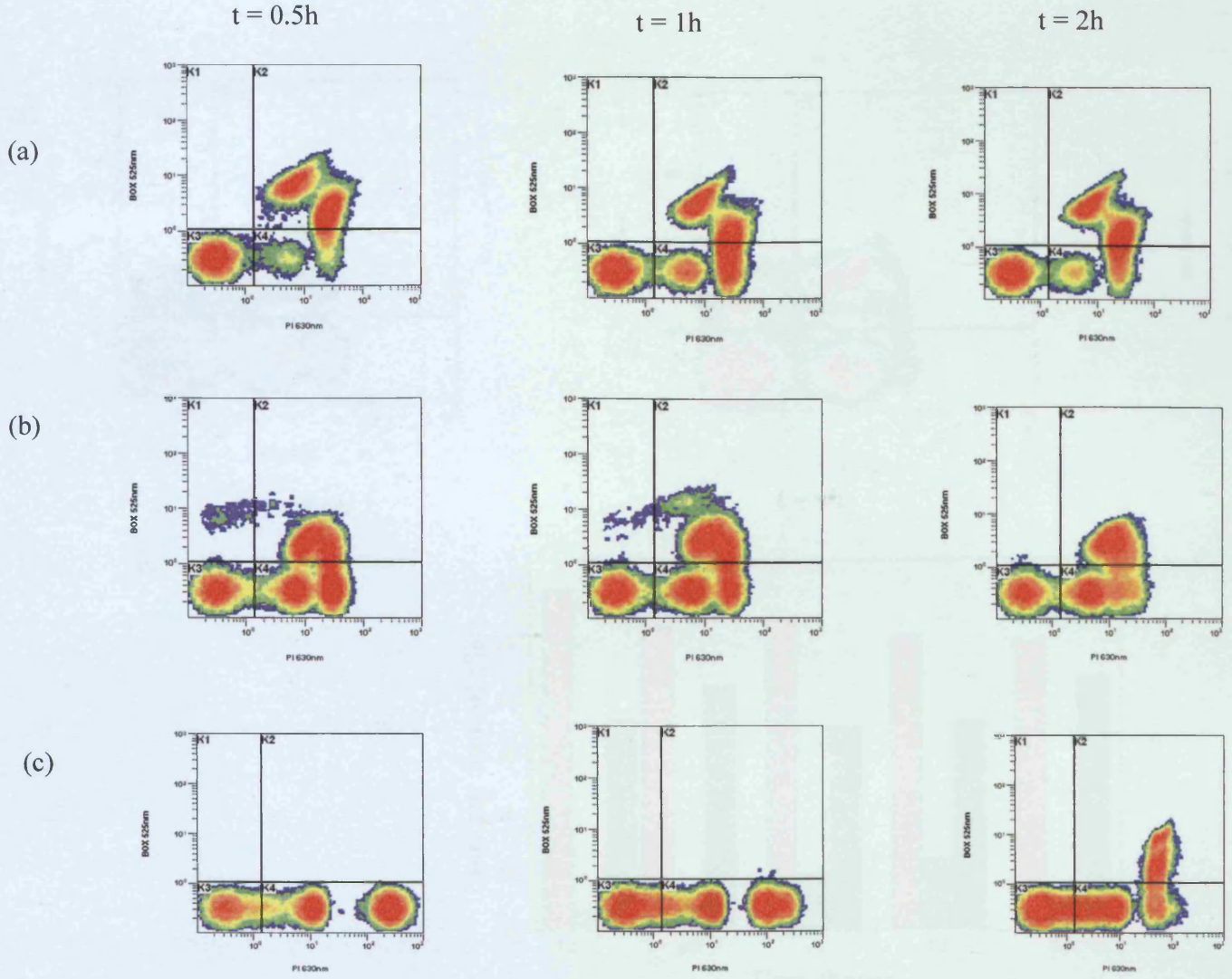


Figure 4.7 Response of *E. coli* TOP10 pQR239 cells to exposure to various miscible co-solvents. (a) 10% v/v ethanol (b) 20% v/v [Emim][TOS] (c) 20% v/v [CABHEM][$MeSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Microbial cultivation was performed as described in Section 2.6 and flow cytometry analysis as in Section 2.8.8.

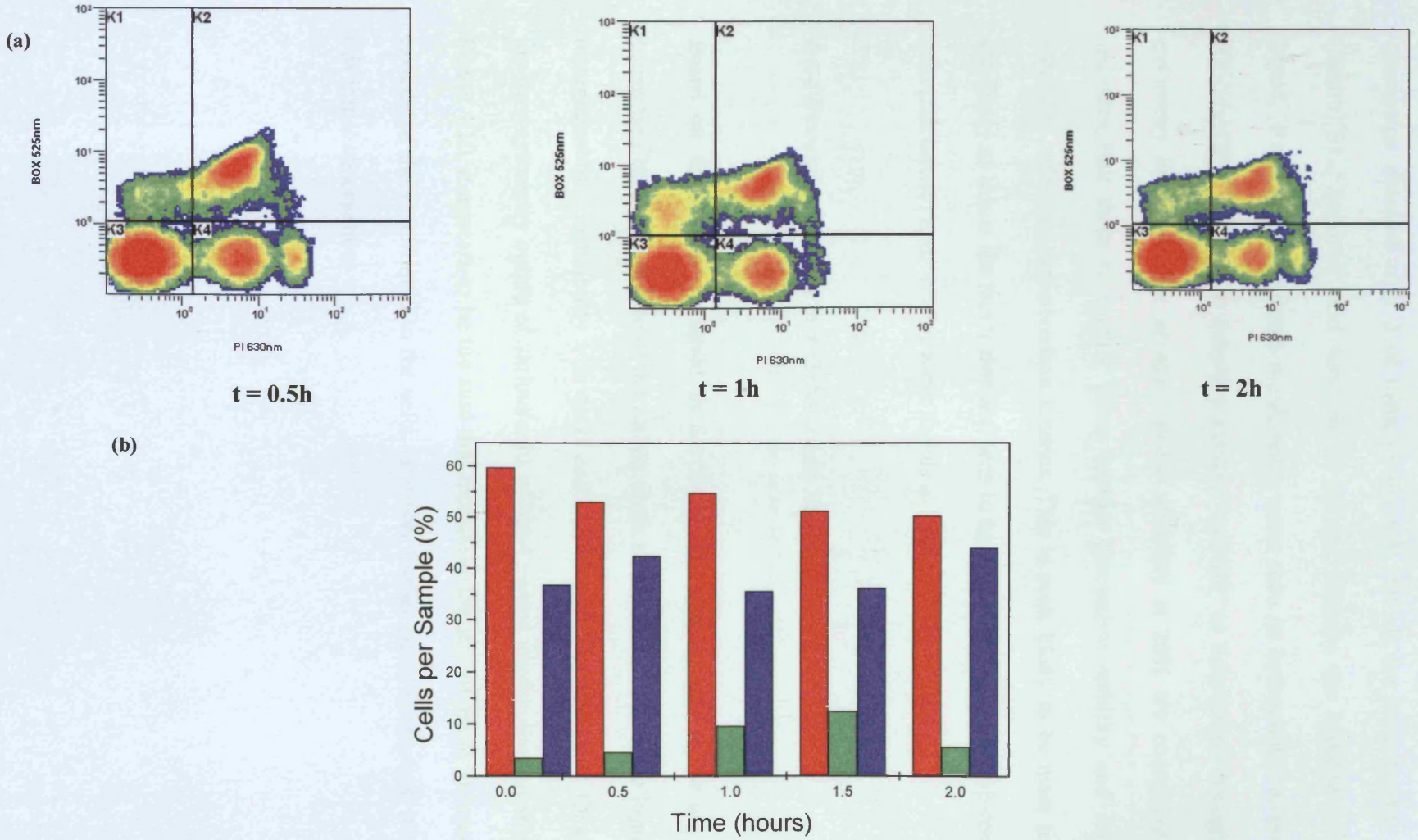


Figure 4.8 (a) Response of *E. coli* TOP10 pQR239 to the immiscible co-solvent [Oc_3MeN][BTA] present at 20% v/v. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. (b) Bar chart of relative cell number of live polarized (red), live depolarized (green) and dead (blue) cells over time. Microbial cultivation was performed as described in Section 2.6 and flow cytometry analysis as in Section 2.8.8.

Many of the ionic liquids examined performed significantly worse than this with no conversion detected with 3 of them (Table 4.3). Of all the ionic liquids tested [Bmim][BF_4] behaves most similarly to ethanol, whereas the hydrophobic ionic liquid, [Bmim][PF_6] performs significantly worse than its hydrophobic counterpart [Oc_3MeN][NTf2]. This is despite a similar "half-life" as determined through flow cytometry and a slightly greater aqueous solubility at 20% v/v co-solvent. This indicates that there is another factor besides biocatalyst stability and substrate solubility affecting bioconversion kinetics. This is most likely to be mass transfer efficiency as this is the factor that was seen to be likely to limit the 6-Br-β-tetralone conversions with hydrophobic ionic liquids in Section 3.4.

4.4.4 Biocatalyst stability and carbon chain length

Based on the results discussed in Section 4.4.2 there appears to be an initial correlation between the size of the carbon chain of the anion of an ionic liquid and its apparent biocompatibility with the *E. coli* TOP10 pQR239 biocatalyst. Observing the flow cytometry output of various salts of butyl methyl imidazolium as shown in Figure 4.10, it appears to be the case that increasing the size of anion increases the toxicity of the ionic liquid to the cells. However further study is required to verify this initial observation.

Co-Solvent	Initial rate ($gL^{-1}hr^{-1}$)	Conversion (% w/w)	$t_{1/2}$ (hr)
[Bmim][BF_4]	0.03	16	0.25
[Emim][TOS]	0.11	30	1
Ethanol	0.07	19.6	0.15
[Bmim][$MDEGSO_4$]	0	0	<0.1
[Bmim][PF_6]	0.01	4	2
[CABHEM][$MeSO_4$]	0	0	<0.1
[Oc_3MeN][NTf2]	0.12	28.3	>2
[Bmim][$OcSO_4$]	0	0	<0.1

Table 4.3 Summary of 1-indanone oxidation data by *E. coli* TOP10 pQR239 in the presence of 20% v/v co-solvent in the case of ionic liquid co-solvents and 10% v/v in the case of ethanol. $t_{1/2}$ indicates the time required for 50% of cells to become depolarized and permeabilised as measured by flow cytometry. Bioconversions were performed as described in Section 2.7.1. Flow cytometry analysis was carried out as described in Section 2.8.8.

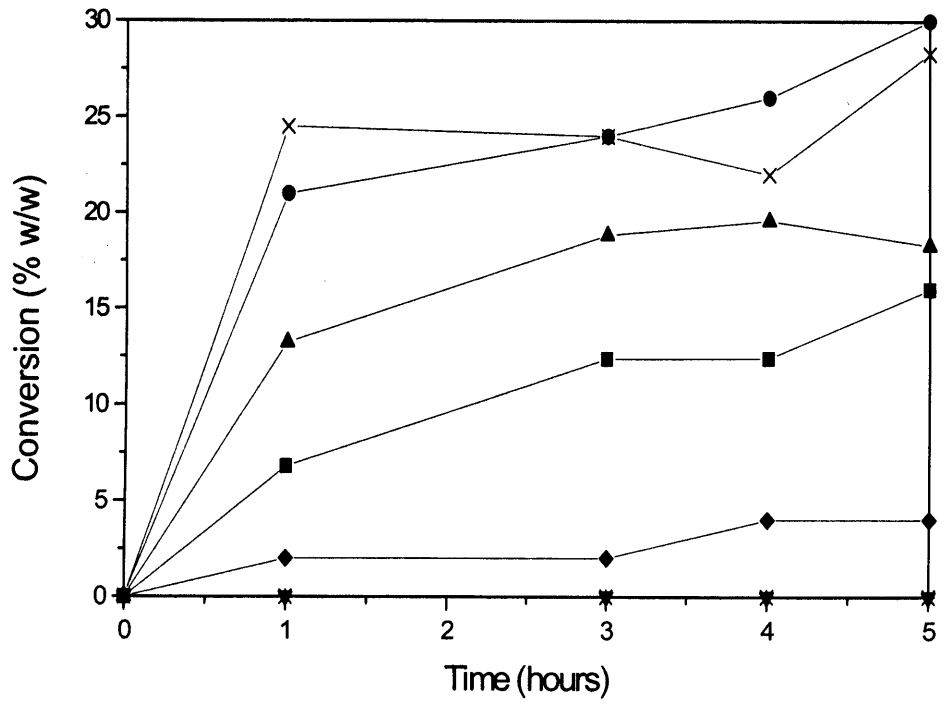


Figure 4.9 Comparison of conversion profiles of 1-indanone to 3,4-dihydrocoumarin by *E. coli* TOP10 pQR239 in the presence of: (■) [Bmim][BF_4] (●) [Emim][TOS] (▲) Ethanol (▼) [Bmim][$MDEGSO_4$] (♦) [Bmim][PF_6] (+) [CABHEM][$MeSO_4$] (x) [Oc_3MeN][NTf2] (*) [Bmim][$OcSO_4$]. Cultivation was carried out as described in Section 2.6 and bioconversions were performed at volume fractions of 10% as described in Section 2.7.1.

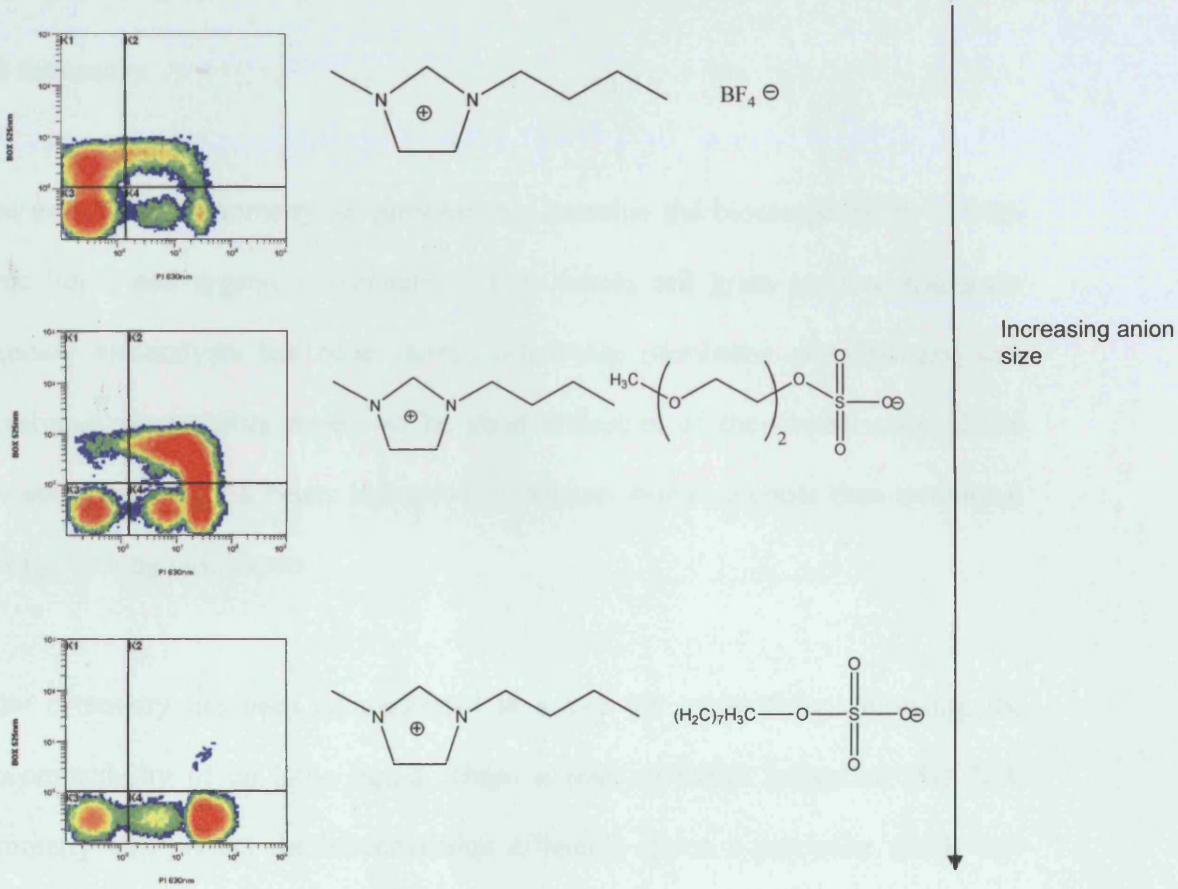


Figure 4.10 Changes in apparent toxicity of ionic liquids to *E. coli* TOP10 pQR239 with anion size. Flow cyometry plot is 30 minutes past exposure in all cases. Experiments were performed at ionic liquid volume fractions of 20% and flow cytometry analysis was as described in Section 2.8.8.

4.5 Summary

The use of flow cytometry to quantitatively examine the biocompatibility of both ionic liquid and organic co-solvents to both whole cell gram positive and gram negative biocatalysts has been shown. Analysing membrane potential and cell membrane permeability appear to be good indicators of the overall state of the biocatalyst and give a better indication of bioconversion potential than traditional colony forming unit counts.

Flow cytometry has been demonstrated as a tool for successfully estimating the biocompatibility of an ionic liquid. There is also an initial indication that flow cytometry will predict the bioconversion efficiency given a particular whole cell biocatalyst and co-solvent combination. However, more data is needed to show this conclusively.

In general, a hierarchy of biocompatibility can be established of immiscible ionic liquids, followed by immiscible organic solvents and then miscible solvents of both types. As expected, gram positive cells are more "solvent tolerant" than gram negative cells, and the mode of toxicity of ionic liquids appears to follow the trend of depolarization of cells (i.e. breakdown of cell metabolism) followed by cell death.

There also appears to be some initial correlation between ionic liquid structure and biocompatibility.

For both of the whole cell biocatalysts examined flow cytometry was successfully used to identify both biocompatible ionic liquids and those ionic liquids that have a high degree of toxicity towards the biocatalyst. For the reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol the ionic liquid [Emim][TOS] was identified through the flow cytometry screen and was used to double conversion of the ketone at a significantly higher rate. In the case of the 1-indanone oxidation a number of ionic liquids were identified two of which gave improved conversions at higher initial rates compared to the conventional ethanol co-solvent.

The great potential of flow cytometry, however, lies in the relative speed and ease of operation compared to traditional viable cell counts. In the cases examined here samples could be taken from the bioconversion, run on a standard flow cytometer and the results obtained in around 10 minutes per sample. The time taken for analysis could be decreased further through the use of automated samplers allowing 32 samples to be loaded and processed at once. As will be described in Chapter 6 there are now also flow cytometers commercially available capable of operating

with standard micro titre plates which open up the possibility of truly high throughput biocompatibility analysis.

5 Isolated enzyme bioreductions of ketones*

5.1 Introduction and aims

The reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by whole cell biocatalysts in the presence of ionic liquids has been successfully demonstrated in Chapter 3. The reactions are limited to 2 gL^{-1} initial substrate concentration however due to substrate toxicity to the biocatalyst beyond this concentration. Such low initial substrate concentrations are not sufficient for industrial application of biocatalysis to chiral alcohol synthesis. Isolated enzyme systems can provide processes with the desired substrate concentrations of 50-100 gL^{-1} (Pollard and Woodley, 2007). The issue of co-factor regeneration in this case has been resolved by the availability of coupled enzymes such as formate dehydrogenase or glucose dehydrogenase that recycle the required co-factor such that its availability does not limit the reaction (Kroutil *et al*, 2004; Pollard and Woodley, 2007). An added benefit of isolated enzyme systems is the amenability of such processes to rapid development which is a priority in early pharmaceutical process development (Pollard and Woodley, 2007).

The challenge with respect to isolated enzyme mediated synthesis is the same as for whole cells; the need to provide large quantities of substrate into the aqueous system, through the use of non-aqueous solvents, is traded off with the inhibitory effects of these solvents on the biocatalyst. The aim of this chapter is to

* The results presented in this chapter have been submitted for publication as: Hussain W, Pollard DJ, Truppo M, Lye GJ. (2008) **Enzymatic ketone reductions with co-factor recycling: improved reactions with ionic liquid co-solvents** *J Mol Catal Enzym* in press.

determine the feasibility of carrying out isolated enzyme bioreductions of 6-Br-β-tetralone with coupled enzyme mediated co-factor recycling in the presence of ionic liquid co-solvents. Key objectives of the work presented in this chapter are to:

- Determine the effects of ionic liquid co-solvents on enzyme stability.
- Characterise bioconversion kinetics in a range of ionic liquid and other co-solvents.
- Determine the feasibility of product recovery from ionic liquids.
- Characterise the limiting factors to reduction reactions involving ionic liquids.

5.2 Biocatalyst identification

A library of 66 commercially available enzyme preparations for ketone reductions was tested against the 6-Br-β-tetralone substrate in an automated screen (Section 2.5) and a selection of the results is presented in Table 5.1. It can be seen that the (*S*) enantiomer of interest can be synthesized with excellent selectivity by four of the enzymes each with 100% ee. In contrast, the (*R*) enantiomer does not appear to be so readily formed. When considering the screening data, the enantio-selectivity of the enzyme is generally deemed more critical than the conversion as the latter can be more readily manipulated through altering some of the engineering parameters when performing the conversion. It was found that the alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) was an effective catalyst for the reduction of interest

(Figure 5.1), with a >99% enantiomeric excess of the desired (*S*) enantiomer. The ADH RE system was chosen for further investigation as it was isolated from the same species previously studied in Chapter 3 (*R. erythropolis*). Glucose dehydrogenase 103 was selected as the co-enzyme used for co-factor recycling.

Enzyme	Conversion (% w/w)	Product ee (%)
KRED1	100.0	24.4
KRED4	25.9	54.7
KRED7	22.7	27.9
KRED8	98.9	-37.1
KRED9	42.3	13.3
KRED10	30.9	55.1
KRED11	98.5	29.4
KRED26	100.0	100
KRED27	100.0	-71.8
KRED28	100.0	-71.5
KRED29	69.6	-43.2
KRED30	92.3	6.4
KRED31	47.7	35.6
KRED exp-A1A	25.7	41.9
KRED exp-A1B	100.0	-43.2
KRED exp-A1C	100.0	-45.1
KRED exp-A1D	100.0	-40.8
KRED exp-A1E	74.1	-18.4
KRED exp-A1I	33.3	30.3
KRED exp-A1J	83.8	9.3
KRED exp-A1L	68.7	78.3
KRED exp-A1P	99.1	100
KRED exp-A1T	73.8	-30.0
KRED exp-A1U	50.0	93.5
KRED exp-A1V	36.3	33.3
ADH RE	99.5	100
KRED NADH 102	100.0	100

Table 5.1. Summary of conversions and product ee of enzymes identified from a screen of 66 ketoreductase preparations for the reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol (Figure 5.1). Product ee was calculated for the (*S*) enantiomer. Enzyme nomenclature is as supplied by the manufacturer (http://www.biocatalytics.com/kred.html). Experiments were performed as described in Section 2.5.

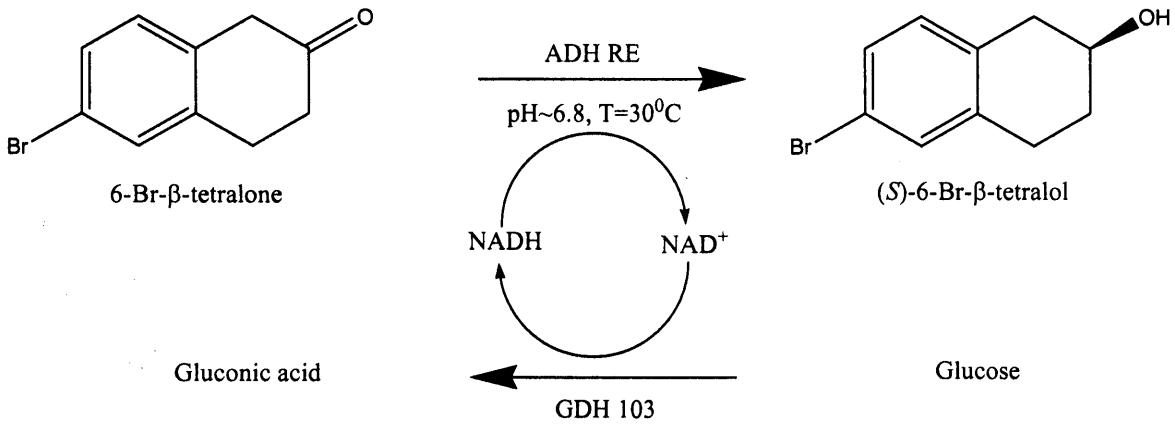


Figure 5.1 The asymmetric reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) and co-factor recycling by the glucose dehydrogenase 103 (GDH 103) mediated oxidation of glucose.

5.3 Substrate solubility screen

One of the primary issues in generating economically viable biocatalytic processes is in effectively charging large titres of substrate to the reaction system (Rozzell, 1999). The compounds of interest are usually sparingly soluble in aqueous systems (<1 gL^{-1}) so the use of a co-solvent is required to boost the delivery of the substrate to the catalyst. In the case of 6-Br-β-tetralone aqueous solubility was found to be very low at less than 0.1 gL^{-1}. Increasing the substrate charge is often achieved through the use of organic solvents that can act as reservoirs of substrate and/or product confining them away from the catalyst until required. The use of solvents in the system however is often detrimental to the catalyst, so using higher volume fractions of solvent to increase substrate delivery further becomes impossible due to inhibition of the biocatalyst (Kim *et al*, 2007). The processing issue becomes a trade off between the need for

effective, concentrated substrate delivery and the need to prolong the activity of the biocatalyst.

A variety of conventional organic solvents with a range of miscibilities with water and polarities were initially screened for solubility of 6-Br-β-tetralone, and some of these are shown in Table 5.2. Solvents with higher polarities, such as methanol, ethanol and iso-propyl alcohol, were found to have relatively poor solubility of the substrate (<75 gL^{-1}), whereas lower polarity solvents dissolved the substrate much more readily. Of these, toluene, DMSO, THF and DMF were found to dissolve the substrate to >500 gL^{-1} in the pure solvent. The partitioning of solute to the conjugate aqueous phase however was much weaker, with only toluene delivering >1 gL^{-1} substrate to the aqueous phase at 10% v/v co-solvent fraction. Even at much higher volume fractions these non-polar solvents failed to deliver >5 gL^{-1} substrate to the aqueous phase.

The 11 ionic liquids screened gave solubilities of the same order of magnitude as polar organic solvents, though solubility levels were often greater than 100 gL^{-1} (Table 5.2). There was no readily discernable difference in behavior between the miscible and immiscible ionic liquids. However, in delivery of substrate to the aqueous phase there was a marked contrast between the behavior of ionic liquids compared to organic solvents and between water miscible and water immiscible ionic liquids. In general the use of ionic liquids resulted in the partitioning of much higher quantities of substrate to the aqueous phase compared to organic solvents (Table 5.2). Two of the AmmoEng^{TM} salts and [Bmim][BF_4] for example delivered ~5 gL^{-1} substrate to the aqueous phase at 10% v/v co-solvent

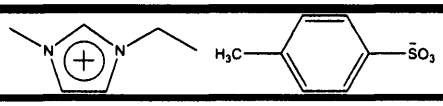
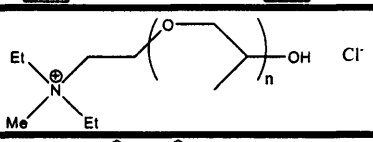
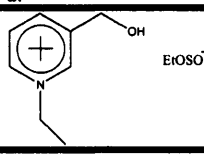
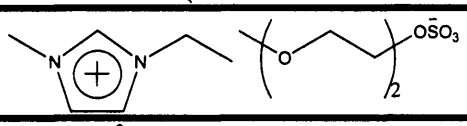
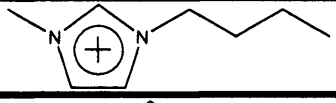
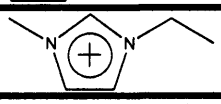
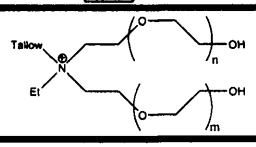
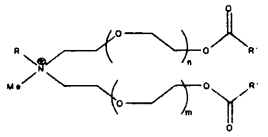
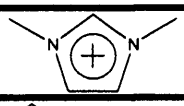
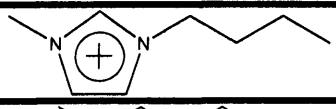
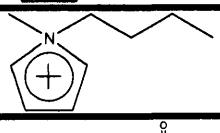
Co-solvent	Structure	Solubility	Aqueous solubility (gL^{-1})			
		(gL^{-1})	5%	10%	20%	50%
[Emim][TOS]		46	3.44	3.09	1.25	11.78
AmmoEng™ 110		64	0.36	0.49	0.63	4.07
[EMP][ES]		65	0.39	0.74	1.67	9.66
EcoEng™ 21M		86	0.97	0.55	3.06	6.89
[Bmim][PF_6]		105	0.17	0.18	0.23	0.32
EcoEng™ 212		121	2.04	0.54	1.30	6.06
AmmoEng™ 102		126	3.49	5.97	18.62	29.78
AmmoEng™ 120		126	4.01	10.76	19.26	55.90
EcoEng™ 1111P		138	3.04	1.16	2.77	32.20
[Bmim][BF_4]		139	0.41	4.97	1.82	62.94
[BMP][NTf2]		164	0.03	0.21	0.14	0.80
DMSO		>500	0.22	0.53	0.95	1.50
THF		>500	0.20	0.32	0.74	3.78
Toluene		>500	2.15	1.00	1.58	4.60
DMF		>500	0.30	0.63	0.81	5.27

Table 5.2 6-Br-β-tetralone solubility in a library of ionic liquids and selected solvents. Solubility refers to the saturation solubility of substrate in 100% v/v co-solvent. Aqueous solubility refers, in the case of miscible co-solvents to the equilibrium saturation concentration of substrate in the co-solvent mixture. In the case of immiscible co-solvents aqueous solubility refers to the equilibrium saturation concentration of substrate in the conjugate aqueous phase. % values refer to the volume fraction of co-solvent to buffer.

fraction compared to less than 1 gL^{-1} for other ionic liquids and for the organic solvents considered.

As ionic salts, ionic liquids possess a much higher polarity than the organic solvents described (Carmichael and Seddon, 2000; Reichardt, 2004) and so are able to dissolve this poorly aqueous soluble substrate more readily in the presence of water. The two immiscible ionic liquids, [BMP][NTf2] and [Bmim][PF_6] show poorer aqueous substrate solubility than even the organic solvents. This indicates that substrate mass transfer from these co-solvents will potentially be an important factor in determining their efficacy to deliver substrate in the aqueous phase containing the biocatalyst.

5.4 Effect of ionic liquids on enzyme stability

The other issue of interest from the standpoint of bioprocess design is enzyme activity. The ADH RE identified in Section 5.2 and GDH 103 were subsequently screened in a library of ionic liquid and organic co-solvents for enzyme stability. Measured half-lives of ADH RE and GDH 103 are summarized in Table 5.3 when determined at 10% v/v co-solvent volume fraction.

ADH RE appeared stable at 30°C in the presence of up to 20% v/v DMSO for 48 hours, with only 30% loss of activity. In contrast, toluene was much more detrimental with less than 5% residual activity after 48 hours in the presence of 10% v/v co-solvent.

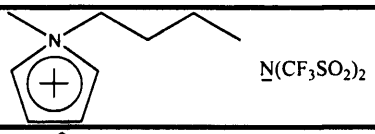
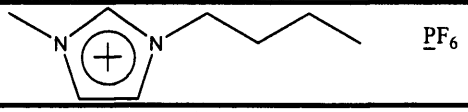
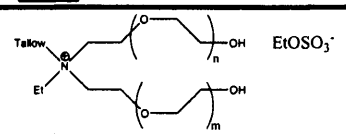
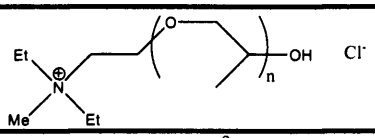
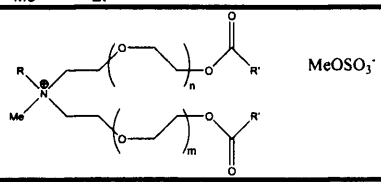
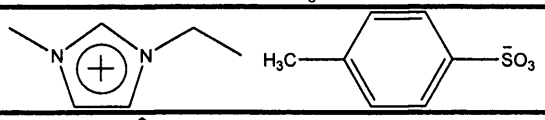
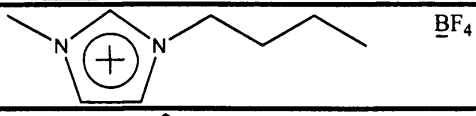
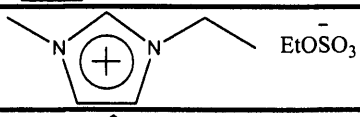
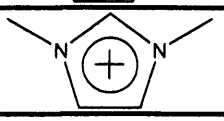
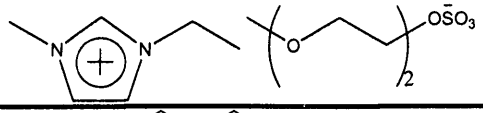
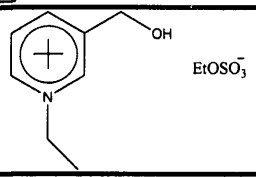
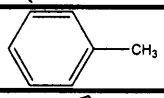
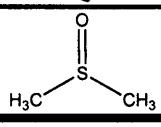
Co-solvent	Structure	Half-life, $t_{1/2}$ (hours)	
		ADH RE	GDH103
None		78	128
[BMP][NTf2]		266	>300
[Bmim][PF_6]		135	220
AmmoEng^{TM} 102		12	>300
AmmoEng^{TM} 110		77	>300
AmmoEng^{TM} 120		40	170
[Emim][TOS]		82	41
[Bmim][BF_4]		45	239
EcoEng^{TM} 212		147	>300
EcoEng^{TM} 1111P		182	108
EcoEng^{TM} 21M		22	97
[EMP][ES]		144	27
Toluene		10	5
THF		<3	25
DMSO		231	34

Table 5.3 Half life of alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) and glucose dehydrogenase 103 (GDH 103) in a range of co-solvents (10% v/v) at 30^{0}C and pH 7. None represents 100% v/v 100 mM potassium phosphate dibasic buffer. Experiments were performed as described in Section 2.6.

This is in contrast to enzyme in 100% buffer (pH 7.0) where 65% of the original activity was retained after 48 hours. Seven out of the eleven ionic liquids screened (Table 5.3) offered an advantage toward the stability of both ADH RE and GDH 103 compared to the organic solvents tested. In particular the immiscible ionic liquids [BMP][NTf2] and [Bmim][PF_6] where 80% and 78% original activity was maintained respectively over 64 hours compared to only 57% activity retention in buffer. This is in contrast to AmmoEng^{TM} 102 and EcoEng^{TM} 21M where >95% enzyme activity was lost after just 15 hours.

5.5 Bioconversion of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol

Bioconversions in the presence of all the ionic liquids listed in Table 5.3 were subsequently performed in pH stats (working volume 10 mL) at an initial substrate concentration of 50 gL^{-1} to determine which co-solvent would result in the best conversion yield and reaction rate. These pH stat reactors were mixed by a magnetic flea, as described in Section 2.7.2, and visual observation of the reactions indicated effective mixing of the two-phase systems was achieved with good dispersion of the ionic liquid phase into the bulk fluid. A summary of the bioconversion data for selected co-solvents is presented in Table 5.4.

The immiscible ionic liquids [BMP][NTf2] and [Bmim][PF_6] performed the best of all the co-solvent systems analyzed. Enzyme stability in the presence of these two co-solvents is good with residual ADH RE activities after 24 hours of 43% and 28% respectively (Table 5.4). The steady state aqueous solubility of the substrate from [BMP][NTf2] and [Bmim][PF_6] to the conjugate aqueous phase

was measured at 0.21 gL^{-1} and 0.18 gL^{-1} respectively which is much lower than for the miscible co-solvent systems. However, the complete conversions and high initial rates of reaction achieved in the presence of these co-solvents suggest enhanced mass transfer rates of substrate from these ionic liquids as a possible factor. The use of 10% v/v toluene as an immiscible co-solvent significantly out performed all the other organic co-solvent systems studied in terms of conversion (Table 5.4, Figure 5.2), and the residual ADH RE activity was similar to [BMP][NTf2]. In addition to this, the solubility of the substrate after equilibration in the aqueous phase is 1 gL^{-1} suggesting the rate of reaction should be greater than that for [BMP][NTf2]. However the rate is almost one third that of [BMP][NTf2], indicating the solute mass transfer rate may be lower from the toluene phase.

Co-solvent	Initial rate ($g(prod)L^{-1}hr^{-1}$)	Conversion (% w/w)	Residual ADH activity (% w/w)	Substrate solubility (gL^{-1})
None	3.1	100	74	0.1
[BMP][NTf2]	14	100	43	0.2
[Bmim][PF_6]	14	98.5	28	0.2
AmmoEng^{TM} 102	12	100	9.4	6.0
[Emim][TOS]	6.5	89.0	51	3.1
[Bmim][BF_4]	9	99.5	38	1.2
EcoEng^{TM} 1111P	1.2	38.5	66	1.3
Toluene	5.8	100	41	1.0
DMSO	2.3	58.4	42	0.5
THF	2	24.9	0.8	0.3
DMF	3.6	95.8	7.6	0.6

Table 5.4 Summary of initial reaction rates, conversion, residual ADH RE activity and substrate solubility in the ionic liquid screen for the reduction of 50 gL^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol (Figure 5.1). All experiments were performed in the presence of 10% v/v co-solvent as described in Section 2.7. In the case of miscible co-solvents substrate solubility refers to the equilibrium saturation of substrate in the co-solvent mixture. In the case of immiscible co-solvents substrate solubility refers to the equilibrium saturation concentration of substrate in the aqueous phase.

Despite a low aqueous solubility of 6-Br-β-tetralone (<1 gL^{-1}) the reaction carried out with no co-solvent (at an added initial amount of substrate equivalent to 50 gL^{-1}) goes to completion within 24 hours at an initial rate of 10.1 $g(prod)L^{-1}hr^{-1}$ (Figure 5.2). The residual ADH RE concentration after this time was 74% w/w (Table 5.4). Visually, the reaction is seen to begin as a two-phase system of aqueous buffer and solid substrate but as product is synthesised an immiscible oil phase is formed. It is suggested that the dispersed oil and solid phase enhance the mass transfer rate of substrate into the aqueous phase yielding the full conversion observed in 24 hours. The presence of dispersed solid phases in particular is known to enhance gas-liquid mass transfer coefficients in bioreactors (Littlejohns *et al*, 2007). The initial rate of reaction in the presence of [BMP][NTf2] is still greater than with the buffer (which have comparable aqueous solubilities) suggesting that the mass transfer improvements of substrate from the [BMP][NTf2] to the aqueous phase are greater than the effects of the enhancements brought about through the presence of the product oil and solid phases.

In the case of miscible ionic liquid co-solvents, the 10% v/v AmmoEng^{TM} 102 co-solvent system yielded 100% w/w conversion with an initial rate of 12 $g(prod)L^{-1}hr^{-1}$ (Table 5.4, Figure 5.2). Residual ADH RE activity was only 10% of the initial value as the enzyme has been shown to have a very short half life in the presence of this co-solvent (Table 5.3). However the solubility of the substrate is boosted to 6 gL^{-1} facilitating more rapid reactions.

In summary, while there does appear to be a correlation between bioconversion efficiency and parameters such as enzyme activity, aqueous substrate solubility and solute mass transfer efficiency there is still a need to carry out a screen of reaction conditions. Based on the data available reliably predicting which conditions will give the highest rates and yields is not currently feasible. For example, the ionic liquid [Emim][TOS] provides an aqueous solubility of 3.1 gL^{-1} (Table 5.4) and ADH RE half life is around 80 hours (Table 5.3) in the presence of 10% v/v co-solvent, but conversion is limited to <90% w/w at a much lower initial rate of 6.5 g(prod) $L^{-1}hr^{-1}$ when compared to [BMP][NTf2].

5.6 Effect of volume fraction

For immiscible co-solvents the volume fraction of the dispersed phase will determine the total amount of substrate that can be dissolved in the system and the interfacial area available for mass transfer. In the case of [BMP][NTf2], the best ionic liquid found in Section 5.5, there appears to be no readily discernable difference in rate or conversion between 10% and 20% v/v volume fractions while at 50% v/v the measured rate is approximately 60% that of the lower volume fractions (Figure 5.3).

Normally mass transfer would be expected to be better at the increased co-solvent volume fraction (Cesario *et al*, 1997). Interestingly the enzyme stability data shows that there is no significant difference in the rate of ADH RE or GDH 103 degradation between the three systems (Figures 5.3(b) and 5.3(c)). This apparent disparity in reaction rate at high volume fraction may be due to the

influence of the viscosity of the ionic liquid as the viscosity of the pure ionic liquid is two orders of magnitude greater than that of water ($9x10^{-2}$ PaS). All three ionic liquid concentrations were operated at the same stirrer speed (1000 rpm) yet the mass averaged viscosity varied 2-3 fold across the experiment resulting in a 50% change in the Reynolds number. The Reynolds number is an engineering parameter indicating the degree of turbulence within a system and is based on the ratio of inertial forces to viscous forces due to agitation. It is defined as $Re = \rho N d_i^2/\mu$ where ρ is the mass averaged system density, N is the impeller speed, d_i is the impeller diameter and μ the mass averaged system viscosity.

5.7 Effect of impeller Reynolds Number

The effect of Reynolds number on the conversion was further examined at Reynolds numbers of 450 and 900 as determined by the minimum and maximum impeller speeds achievable on the lab scale equipment (Section 2.7.2). Figure 5.4 shows the conversion profiles for two 10% v/v [BMP][NTf2] co-solvent systems, one at a Reynolds number of 450, the other at 900, and a 20% v/v [BMP][NTf2] co-solvent system with a Reynolds number of 450. As expected, at a fixed co-solvent fraction and in a heterogeneous reaction system that is mass transfer limited doubling the Reynolds number doubles the initial rate of reaction. Likewise at a fixed Reynolds number doubling the co-solvent fraction leads to a corresponding increase in the measured rate of reaction due to an increase in the area available for solute mass transfer. These results confirm that

mass transfer is the limiting factor for the ionic liquid bioconversions studied in this work.

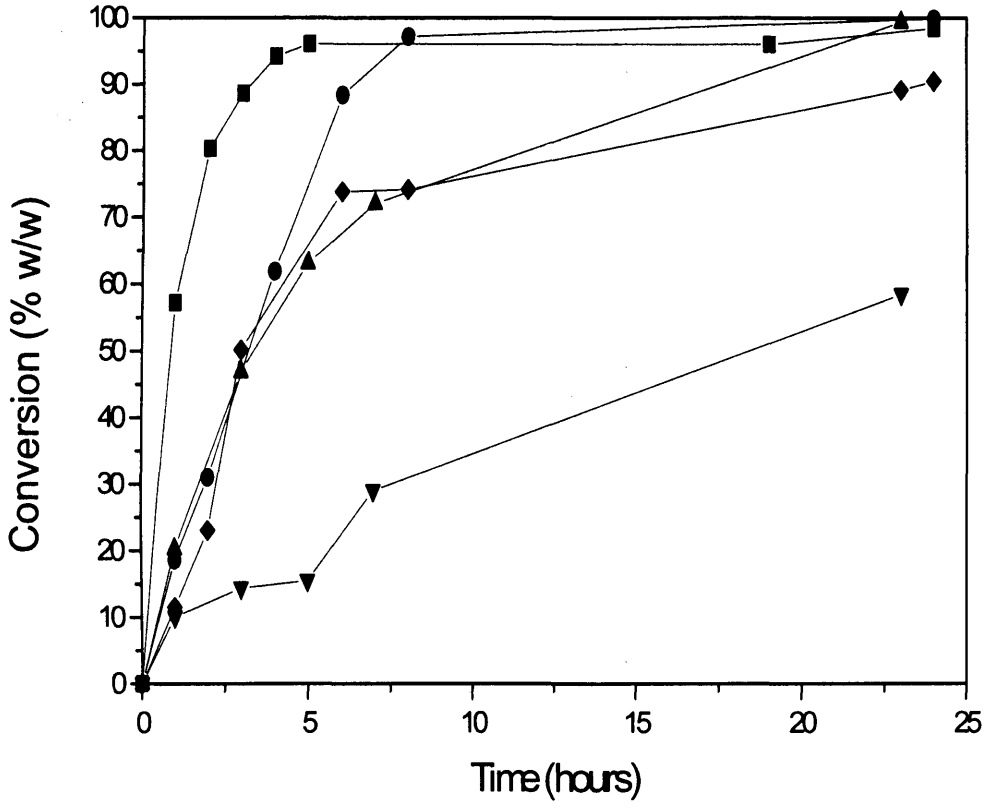


Figure 5.2 Comparison of the conversion kinetics of 50 gL^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by ADH RE in the presence of 10% v/v co-solvent: (■) [BMP][NTf2], (●) AmmoEng™ 102, (▲) toluene (▼) DMSO and (♦) with no co-solvent (buffer only). Reactions were carried out in Multimax™ miniature reactors with 30 mL working volume at 30°C and pH 6.8 ± 0.1 as described in Section 2.7.2.

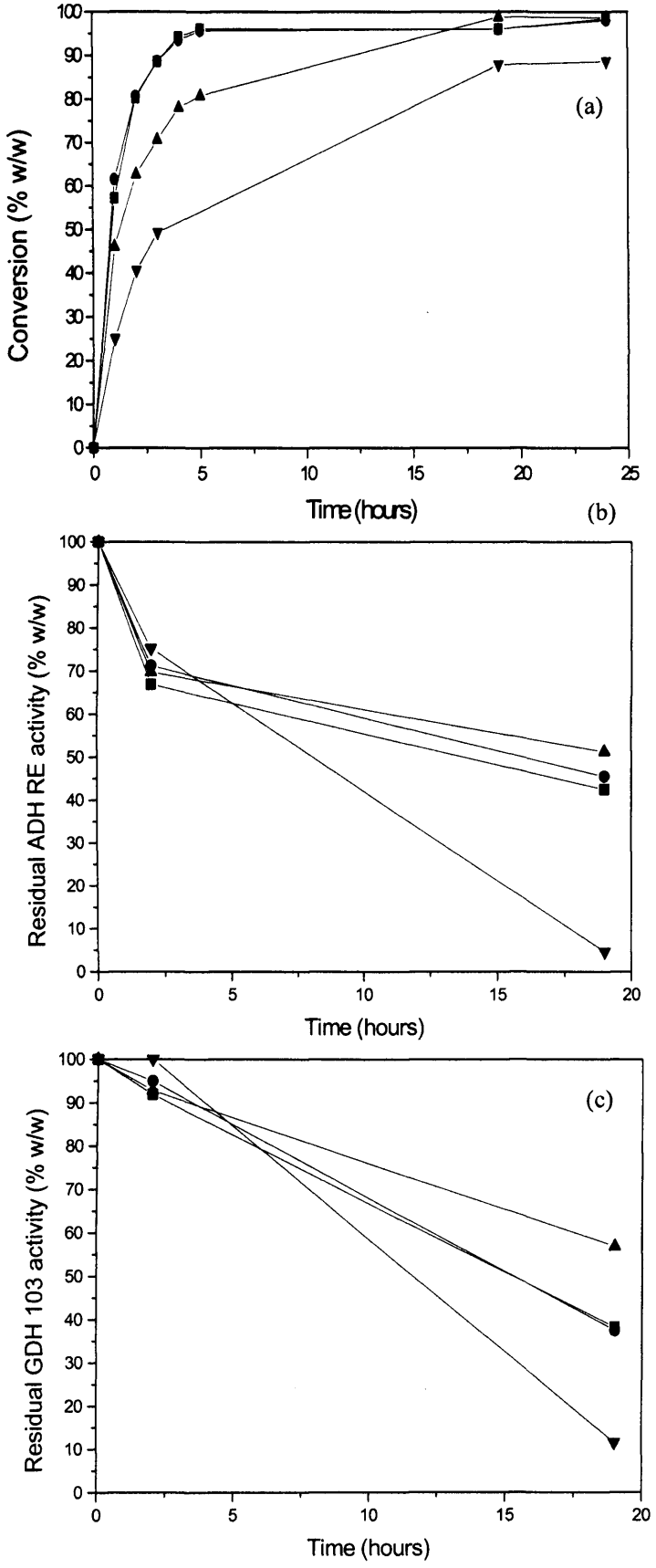
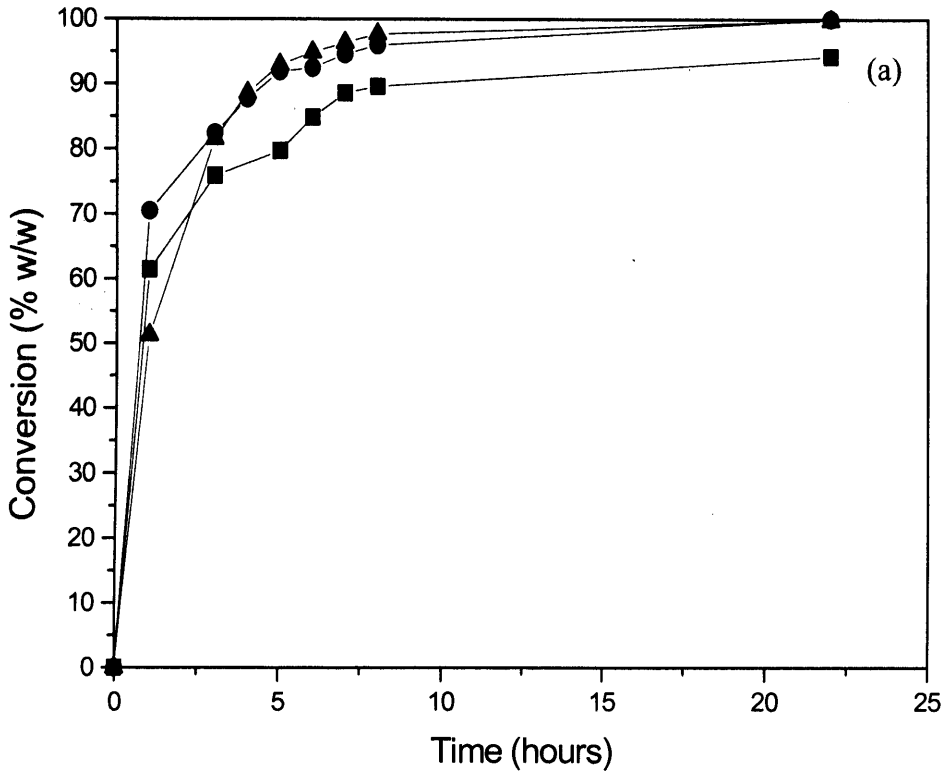


Figure 5.3 Effect of co-solvent volume fraction on the conversion of 50 gL^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by ADH RE in a range of co-solvents: (a) conversion kinetics (b) residual ADH RE activity (c) residual GDH 103 activity. Experiments performed in the presence of: (■) 10% v/v [BMP][NTf2], (●) 20% v/v [BMP][NTf2], (▲) 50% v/v [BMP][NTf2] and (▼) 10% v/v toluene. Reactions were carried out in Multimax™ miniature reactors as described in Section 2.7.2.



(b)

Re	450	900	450
[BMP][NTf2] (% v/v)	10	10	20
Initial rate ($g(prod)L^{-1}hr^{-1}$)	6.8	14.1	14.3

Figure 5.4 Effect of impeller Reynolds number and dispersed phase volume fraction on initial rate of reaction for conversion of 50 gL^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by ADH RE. (a) Conversion kinetics of: (■) 10% v/v [BMP][NTf2], Re = 450, (●) 10% v/v [BMP][NTf2], Re = 900, (▲) 20% v/v [BMP][NTf2], Re = 450. (b) Calculated initial rates of reaction. Reactions were carried out in Multimax™ miniature reactors as described in Section 2.7.2.

5.8 Effect of enzyme concentration

Due to the high cost of enzyme preparations, industrial enzymatic processes are rarely run such that mass transfer is the limiting factor. Enzyme concentration is a more restrictive constraint (Pollard *et al*, 2006*a*) and so was also examined. At a fixed Reynolds number of 450 and 10% v/v [BMP][NTf2] co-solvent, various initial ADH RE concentrations (0.5-5.0 gL^{-1}) were used to determine the enzyme concentration at which mass transfer becomes rate limiting (GDH 103 concentration was maintained at 0.5 gL^{-1} for all experiments). As expected increasing initial enzyme concentration increases the rate of reaction and the time to complete conversion is reduced. The results, shown in Figure 5.5, indicated that solute mass transfer first becomes rate limiting at an initial ADH RE concentration of ~2 gL^{-1}.

5.9 Effect of temperature on conversion kinetics

It was shown that the ADH RE stability at 40°C was not significantly different from stability at 30°C (Table 5.3). Consequently, the 1 gL^{-1} initial ADH RE experiment originally described in Section 5.8 was compared to a similar experiment run at 45°C. From Figure 5.6(a) it can be seen that the initial rate of reaction was much greater (approximately double) at the higher temperature but this rate diminished sharply after 2 hours due to a much more rapid decrease in ADH RE activity at this higher temperature.

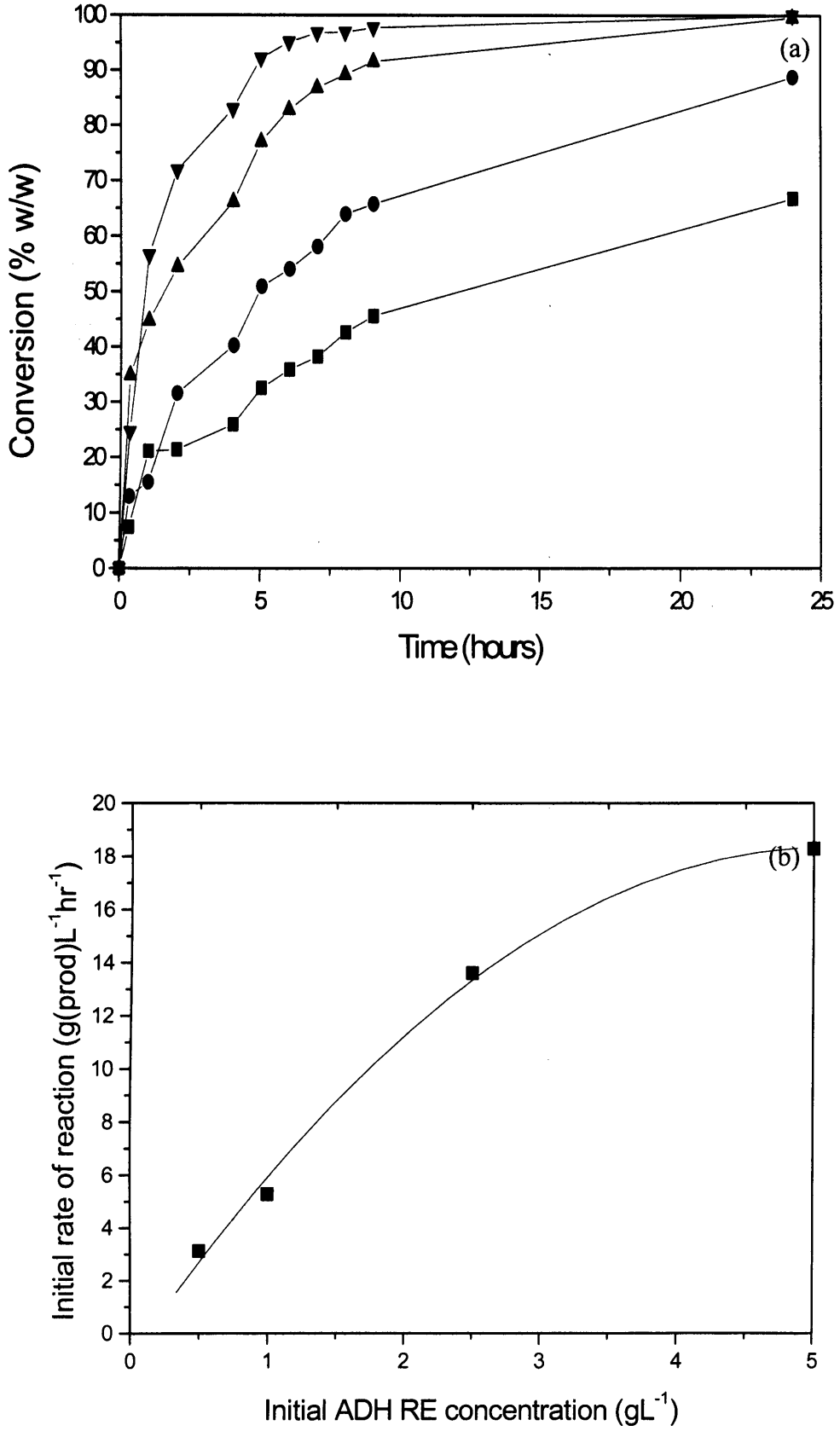


Figure 5.5 Effect of initial ADH RE concentration on the conversion kinetics of 50 gL^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol (a) Comparison of % conversion kinetics at initial ADH RE concentrations of: (■) 0.5 gL^{-1}, (●) 1.0 gL^{-1}, (▲) 2.5 gL^{-1} and (▼) 5.0 gL^{-1}. (b) Initial rate of reaction against initial enzyme concentration. Solid line fitted by second order polynomial regression (R^2 = 0.996). Experiments were performed as described in Section 2.7.2.

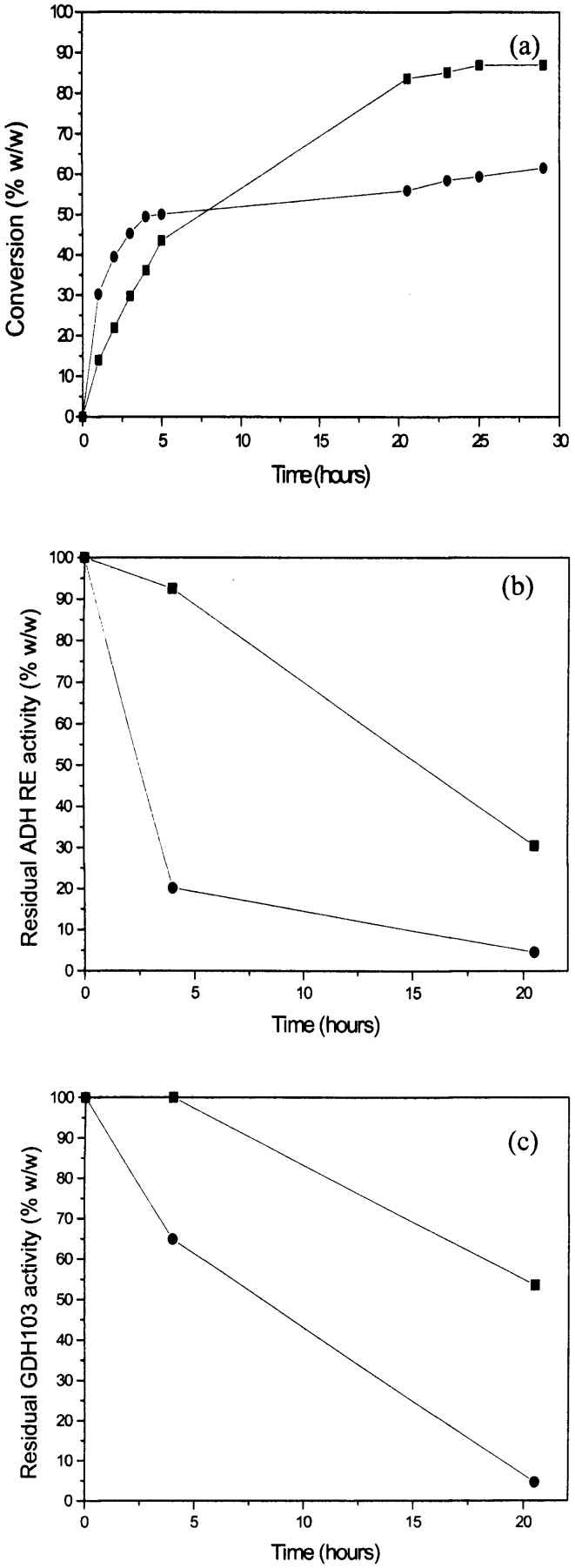


Figure 5.6 Effect of temperature on the conversion of 50 gL^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol at an initial ADH RE concentration of 1 gL^{-1}. (a) Comparison of conversion kinetics, (b) residual ADH RE activity, (c) residual GDH 103 activity at: (■) 30°C (●) 45°C. Experiments were performed as described in Section 2.7.2.

This apparent disparity between the stability data observed between enzyme in buffer and co-solvent in vials (in thermomixers) and the reaction vessel is attributed to the harsher mixing conditions in the reaction vessel.

5.10 (*S*)-6-Br-β-tetralol recovery

A study of the partition coefficient for 6-Br-β-tetralone between [BMP][NTf2] and various organic solvents showed that toluene gave the best extraction of product from the ionic liquid. A simple isolation procedure was feasible whereby the product in the immiscible ionic liquid can be separated from the aqueous phase by centrifugation followed by three half-volume extractions with toluene from the ionic liquid (~60% w/w average extraction efficiency). The product was then isolated from the toluene by vacuum evaporation of the solvent yielding 88% w/w product with an enantiomeric excess of >99% in favour of the desired (*S*) enantiomer.

5.11 Acetophenone reduction

5.11.1 Introduction

A further example of an asymmetric ketone reduction to which biocatalysis is a desirable route of formation is the reduction of 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol. The reaction is described in Figure 5.7. Reduction of multi-substituted pro-chiral acetophenones for pharmaceutical synthesis has been previously reported with whole cell

biocatalysis (Patel *et al*, 2004*b*) and by isolated enzymes (Pollard *et al*, 2006*b*). In these cases the acetophenones were either highly soluble in aqueous media or the reaction conditions could be manipulated so that the substrate was readily converted by the biocatalyst of choice. In the particular case of 4'-Br-2,2,2-trifluoroacetophenone the substrate is soluble in aqueous media to concentrations sufficient to drive the reaction but was found to be inhibitory towards the biocatalyst. The application of room temperature ionic liquids is investigated here to address this bottleneck in chiral alcohol synthesis.

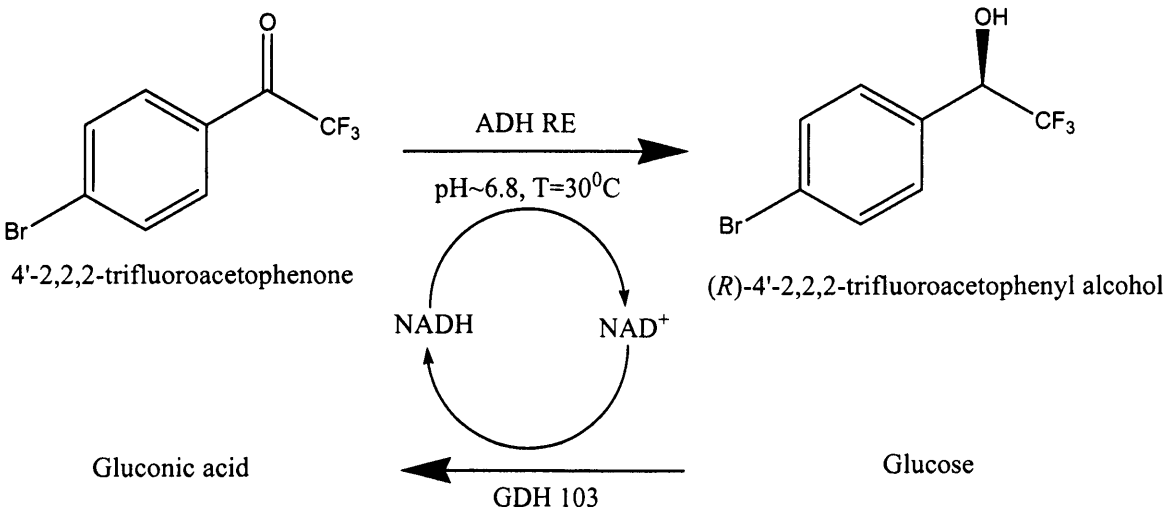


Figure 5.7 The asymmetric reduction of 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4-Br-2,2,2-trifluoroacetophenyl alcohol by ADH RE and co-factor recycling by the GDH 103 mediated oxidation of glucose.

5.11.2 Biocatalyst identification

Enzymes suitable for the reduction of 4'-Br-2,2,2-trifluoroacetophenone to (*R*) 4'-Br-2,2,2-trifluoro phenyl ethanol (Figure 5.7) were initially identified by

screening the substrate against the commercially available ketone reduction library used in Section 5.2. Enzymes yielding both product enantiomers were found. The alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) gave the desired (*R*) enantiomer with an enantiomeric excess (ee) > 99% while the ketoreductase KRED 101 gave the (*S*) enantiomer. Both of these enzymes used a second enzyme, glucose dehydrogenase 103 (GDH 103), to regenerate the cofactor (NADH).

5.11.3 Bioconversion of 4'-Br-2,2,2-trifluoroacetophenone to (R)-4'-2,2,2-trifluoroacetophenyl alcohol

All bioconversions were investigated at an industrially relevant initial substrate concentration of 50 gL^{-1}. For bioconversions in buffer (Figure 5.8(a)) or with 10% v/v of miscible organic co-solvent such as methanol, DMSO or THF only 10% conversion was achieved. This was not due to the low solubility of the substrate as the solubility in buffer alone is > 6 gL^{-1}. Rather, the poor conversion was due to rapid deactivation of both enzymes as shown by the activity profiles for ADH RE and GDH 103 in Figure 5.8(b) and 5.8(c) respectively. Separate enzyme stability studies in buffer with 10 gL^{-1} substrate (the aqueous solubility limit) showed an enzyme half life of < 2 hours which is severely reduced compared to enzyme stability measured in buffer only where the half life is 78 hours for ADH RE and >300 hours for GDH 103 (Table 5.3). Biphasic systems with organic solvents gave some improvement to conversion as for example up to 20% w/w conversion in the presence of 10% v/v toluene (Figure 5.8(a)). The toluene acted as a reservoir for the substrate so the aqueous substrate

concentration the enzymes were exposed to was lowered to around 4 gL^{-1}. As shown in Figure 5.8(b) and 5.8(c) the presence of 10% v/v toluene reduced the deactivation on the enzyme such that a low level enzyme activity was detectable even after 24 hours.

Bioconversions in the presence of ionic liquid such as the immiscible [BMP][NTf2] showed rapid reaction rates with complete conversion of an initial 50 gL^{-1} substrate concentration in less than 10 hours (Figure 5.8(a)). The use of AmmoEng^{TM} 102 also led to improved reactions. The calculated initial rate of reaction with no co-solvent was approximately 3 $g(prod)L^{-1}hr^{-1}$ which almost doubled in the presence of 10% v/v AmmoEng^{TM} 102 to 5.5 $g(prod)L^{-1}hr^{-1}$ and doubled again to 12.3 $g(prod)L^{-1}hr^{-1}$ in the presence of 10% v/v [BMP][NTf2]. In the presence of a number of ionic liquids, especially [BMP][NTf2], the ADH RE half-life was markedly improved as shown in Table 5.3, and its residual activity was less affected by the presence of substrate as shown in Figure 5.8(b). A total of four of the ionic liquids tested gave conversion improvements over the best organic co-solvent tested and three of these significantly protected the enzyme from the inhibitory effects of the substrate (Table 5.5).

In Section 5.4 it was seen that the presence of some ionic liquids enhanced the stability of ADH RE. Among these was the ionic liquid [BMP][NTf2]. In the presence of this ionic liquid at 10% volume fraction the half life of ADH RE increased to 266 hours and that of GDH 103 to >300 hours. This is in contrast to buffer alone where half life of the ADH RE and GDH 103 was 78 and 128 hours respectively. The increase in enzyme stability in the presence of [BMP][NTf2]

however is not sufficient to account for the large increases in initial activity observed in Figure 5.8(a), suggesting a further enhancement of other physical parameters may be in operation such as that observed for the 6-Br-β-tetralone substrate in Section 5.4.

Co-solvent	Initial rate ($g(prod)L^{-1}hr^{-1}$)	Conversion (% w/w)	Residual ADH activity (% w/w)	Substrate solubility (gL^{-1})
None	3.1	5.6	0	6.5
[BMP][NTf2]	12	100	40	5.1
[Bmim][PF_6]	11	99.6	47	3.8
AmmoEng™ 102	5.5	75.8	4	14
[Emim][TOS]	2.5	46.1	46	8.2
[Bmim][BF_4]	0.1	0	0	6.3
EcoEng™ 1111P	0.1	0.8	0	11
Toluene	6.1	20.8	1	8.2

Table 5.5 Summary of initial reaction rates, conversion, residual ADH RE activity and substrate solubility in the ionic liquid screen for the reduction of 50 gL^{-1} 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol (Figure 5.7). All experiments were performed in the presence of 10% v/v co-solvent as described in Section 2.7. In the case of miscible co-solvents substrate solubility refers to the equilibrium saturation of substrate in the co-solvent mixture. In the case of immiscible co-solvents substrate solubility refers to the equilibrium saturation concentration of substrate in the aqueous phase.

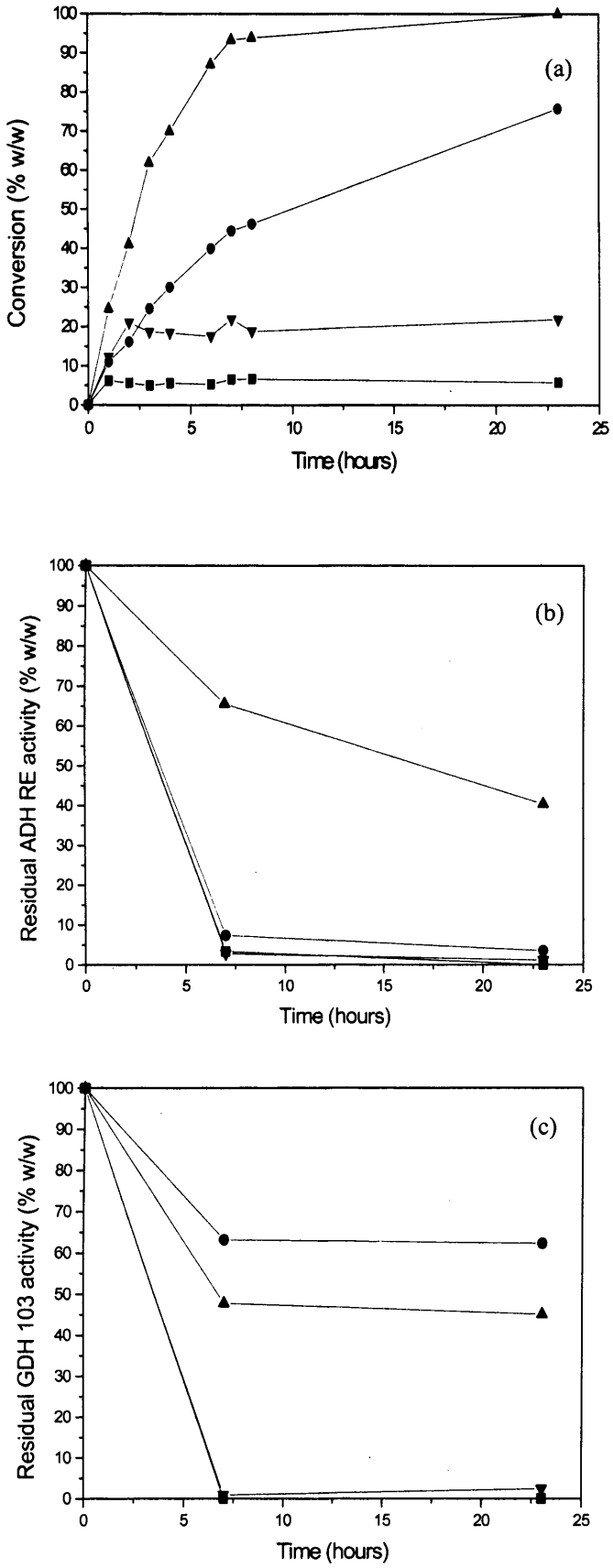


Figure 5.8: Comparison of the conversion kinetics of 50 gL^{-1} 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-2,2,2-trifluoroacetophenyl alcohol by ADH RE in different co-solvent systems: (a) conversion kinetics (b) residual ADH RE activity (c) residual GDH 103 activity. Experiments performed in the presence of 10% v/v co-solvent: (▲) [BMP][NTf2], (●) AmmoEng™ 102, (▼) toluene and (■) with no co-solvent (buffer only). Reactions were carried out in Multimax™ miniature reactors with 30 mL working volume at 30^0C and pH 6.8 ± 0.1 as described in Section 2.7.2.

5.11.4 Effect of enzyme concentration

Experiments in which the initial ADH RE concentration was reduced from 1 gL^{-1} to 0.5 gL^{-1} (GDH 103 concentration was also reduced to 0.5 gL^{-1}) still allowed for 100% w/w conversion of 50 gL^{-1} 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol within 24 hours. The use of 0.1 gL^{-1} ADH RE, although found to be feasible, resulted in too low a rate of reaction to give full conversion within the lifetime of the enzyme in the system. In this case enzyme concentration became the limiting factor as evidenced by the linear conversion rates through the majority of the reaction course.

5.11.5 Ionic liquid re-use

In order to investigate ionic liquid re-use a 0.25 gL^{-1} ADH RE, 50 gL^{-1} initial substrate concentration and 15% v/v [BMP][NTf2] experiment was performed at 30°C in a pH stat (as described in Section 2.6) for ~28 hours, converting ~95% w/w 4'-Br-2,2,2-trifluoroacetophenone to alcohol. In order to isolate the product, this mixture was taken, centrifuged, and the water layer removed. The product and remaining substrate was then extracted from the ionic liquid layer by 4 washes with diethyl ether until >99% w/w of product was recovered (as determined by reverse phase HPLC). It was apparent by eye that some of the ionic liquid had been absorbed into the diethyl ether layer so fresh ionic liquid was added (~0.25 mL giving a total ionic liquid volume of 1.5 mL) to replenish the system.

A second bioconversion (shown in Figure 5.10) in which the recovered ionic liquid was used with fresh enzyme was then performed under the same reaction conditions and compared to a simultaneously run fresh ionic liquid experiment. The recovered ionic liquid profile mirrored the fresh ionic liquid profile very well, but the rate always remained slightly lower than in the fresh ionic liquid case. In the case of the recovered ionic liquid 85% w/w conversion was achieved compared to 92% w/w conversion in the case of fresh ionic liquid.

5.11.6 (R)-4'-Br-2,2,2-trifluoroacetophenyl alcohol recovery and yield

The isolated product from the original reaction with [BMP][NTf2] was recovered by rotary vacuum evaporation of the diethyl ether leaving a viscous oil identified as (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol by reverse phase HPLC (Section 2.8.1) and SFC analysis (Section 2.8.3). The overall yield of recovered product was 85% w/w with an ee of >99% in favour of the desired (*R*) enantiomer.

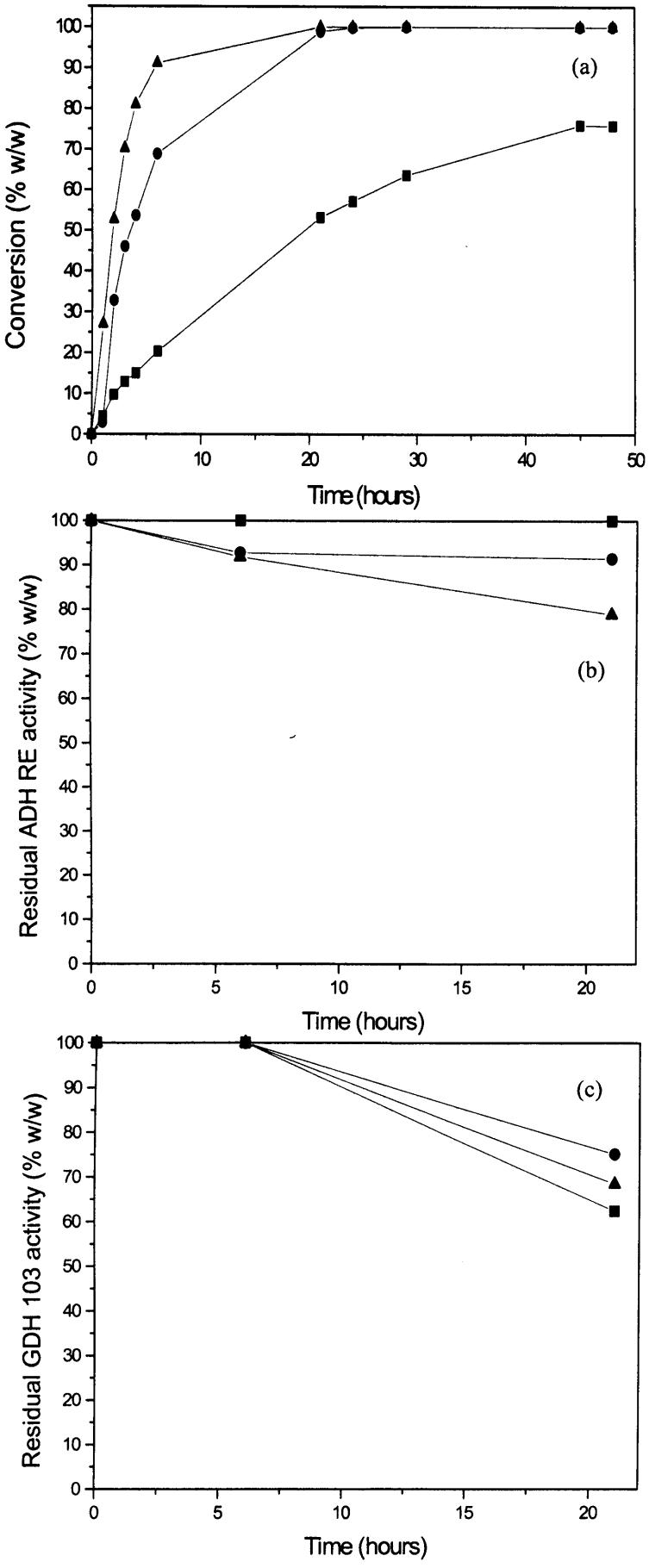


Figure 5.9: Effect of initial ADH RE concentration on the conversion kinetics of 50gL^{-1} 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2 trifluoroacetophenyl alcohol with 10% v/v [BMP][NTf2] co-solvent. (a) Conversion kinetics, (b) residual ADH RE activity, (c) residual GDH 103 activity with: (▲) 1 gL^{-1} (●) 0.5 gL^{-1} (■) 0.1 gL^{-1} initial ADH RE concentration. Experiments were performed as described in Section 2.7.2.

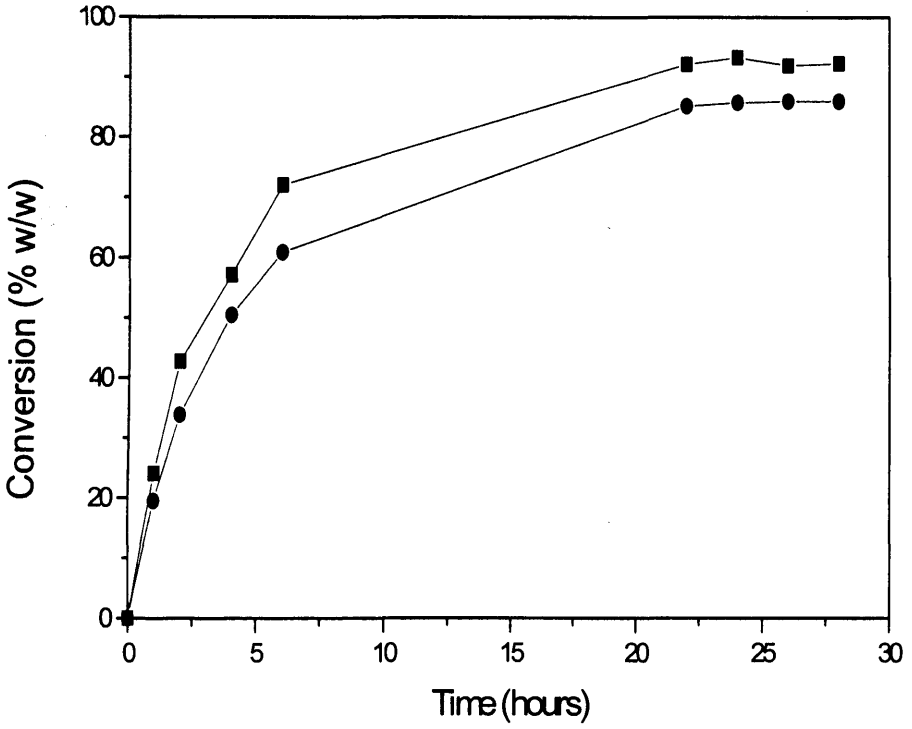


Figure 5.10: Example of ionic liquid re-use. Conversion kinetics of 4'-Br-2,2,2-trifluoroacetophenone reduction to (*R*)-4'-Br-2,2,2-trifluoroacetophenol in the presence 15% v/v (■) fresh (●) recycled [BMP][NTf2] as co-solvent. Ionic liquid was recovered as described in Section 2.10. Bioconversions were performed in pH stats (10 mL working volume) as described in Section 2.7.2.

5.12 Lewis cell mass transfer study

The suggestion of improved substrate mass transfer observed in the 10% v/v [BMP][NTf2] system for the ADH mediated reduction of 6-Br-β-Tetralone to (*S*)-6-Br-β-Tetralol (Section 5.5) was investigated further in a Lewis Cell, as described in Section 2.10 (Lye and Stuckey, 2001). A Lewis Cell provides a constant and defined interfacial area between two immiscible liquid phases and allows for accurate determination of changes in solute concentration in the two phases over time under controlled hydrodynamic conditions. Theoretically, operation of the cell requires equal Reynold's numbers between the two phases (Lye and Stuckey, 2001). However, due to the large differences in density and viscosity between the ionic liquid and buffer, and mechanical limitations of the Lewis Cell available it was not possible to achieve equal Reynold's numbers in each phase. Consequently experiments were run based on varying the ionic liquid phase Reynold's number (from 120 to 430) while keeping that of the aqueous phase constant at 430. Despite these limitations, the results shown in Figure 5.11 indicate a clear advantage in mass transfer of 6-Br-β-tetralone from [BMP][NTf2] to buffer compared to transfer of the substrate from toluene to buffer, even at much reduced Reynold's number for the ionic liquid system, given the equilibrium concentrations from each ionic liquid phase shown in Table 5.2. These results confirm a much enhanced increase (more than 5-fold) in substrate mass transfer rate from ionic liquid to buffer compared to toluene. However, the reason for this enhancement is currently unclear.

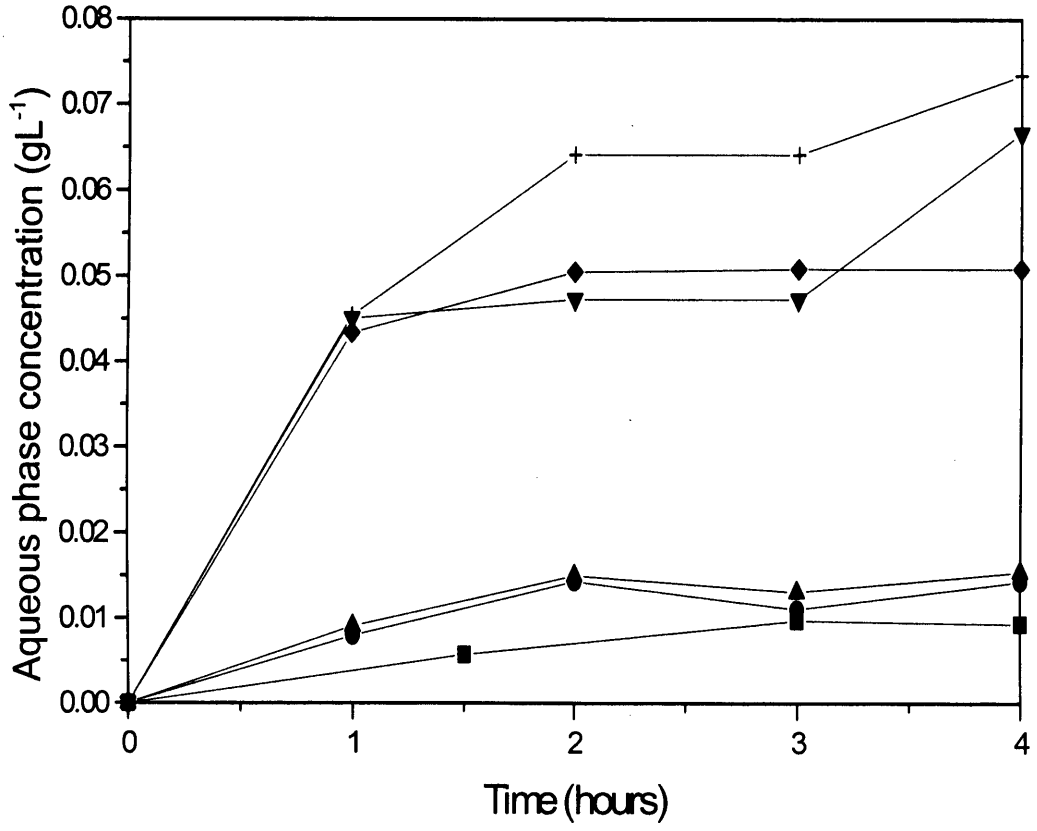


Figure 5.11 Concentration of 6-Br-β-Tetralone in the aqueous buffer phase of Lewis Cell in the presence of toluene with Reynold's number (■) 1320 (●) 3000 (▲) 4500 and [BMP][NTf2] with Reynold's number (▼) 123 (♦) 250 (+) 430. The Lewis Cell was operated as described in Section 2.11.

5.13 Summary

The asymmetric reduction of industrially relevant quantities of prochiral ketones to their corresponding alcohols in the presence of ionic liquid as a co-solvent and with coupled enzyme regeneration of co-factor has been demonstrated. In the reactions studied here the ionic liquid was found not to alter the enantiomeric selectivity of the enzyme.

Use of an immiscible ionic liquid, in particular [BMP][NTf2], led to dramatic improvements in reaction performance. In the case of the reduction of 4'-Br-2,2,2-trifluoroacetophenone the initial rate of reaction in the presence of 10% v/v [BMP][NTf2] increased to 12 g(prod)$L^{-1}hr^{-1}$ with 100% w/w conversion in 24 hours (Figure 5.8(a), Table 5.5). The highest rate achievable with organic solvents was with 10% v/v toluene where only 20% w/w conversion was achieved in 24 hours at an initial rate of ~6 g(prod)$L^{-1}hr^{-1}$ (Table 5.5). Thus, the use of ionic liquids as co-solvents not only improved the rate of reaction but resulted in a previously infeasible reaction becoming potentially industrially viable. In the case of the reduction of 6-Br-β-tetralone use of ionic liquids led to more than a two-fold increase in initial rate of reaction to 14 g(prod)$L^{-1}hr^{-1}$ in the presence of 10% v/v [BMP][NTf2] and [Bmim][PF_6] (Table 5.4). This is in contrast to 10% v/v toluene where the initial rate of reaction was 5.8 g(prod)$L^{-1}hr^{-1}$ and buffer only where the initial rate of reaction was just 3.1 g(prod)$L^{-1}hr^{-1}$ (Table 5.4).

Recovery of the products from the ionic liquids has also been shown to be viable, with little loss in yield and purity. In the case of [BMP][NTf2] separation of the ionic liquid from the aqueous phase where the enzyme resides, proved feasible by centrifugation where a protein precipitate layer forms between the two phases. Extraction of the product from the ionic liquid into organic solvent was rapid and subsequent isolation of the product by rotary vacuum evaporation is a standard technique.

Half life increases to > 250 hours for ADH RE and to >300 hours for GDH 103 at 30°C in the presence of 10% v/v [BMP][NTf2] have also been shown. Previous work on ADH from *Lactobacillus brevis* showed increases of half life to 50 hours under similar conditions in the presence of [Bmim][$(CF_3SO_2)_2N$] (Patel *et al*, 2005). Rates of reaction have also been vastly improved with >95% w/w conversion of 50 gL^{-1} substrate achieved in less than 8 hours in both cases studied here.

Based on the current results and the few examples in the literature (Park and Kazlauskus 2001; Laszlo *et al*, 2002; Hinckley *et al*, 2002; Kaar *et al*, 2003) , there does not at present appear to be a rational way to select ionic liquids for biocatalytic processes based upon their structure or functional groups. Screening of a limited range of ionic liquids has been shown to be effective in a number of cases (Roberts at al, 2004; Lozano *et al*, 2006; Zhang *et al*, 2006). A representative library of ionic liquids can be readily formed for most applications. Of the eleven ionic liquids screened here for the 6-Br-β-tetralone conversion (Section 5.4), five gave rates and conversion equivalent or better than

those of the best organic solvent screened. In the case of the 4'-Br-2,2,2-trifluoracetophenone reduction (Section 5.10.3) four ionic liquids gave improvements over the best organic co-solvent but critically they all facilitated much greater conversion over organic solvents and buffer. Important factors in ionic liquid selection appear to be solubility of substrate in the aqueous phase of heterogeneous phase systems, mass transfer rates of substrate from immiscible ionic liquids into the aqueous phase and biocatalyst stability in the aqueous/ co-solvent mixture.

The enhanced mass transfer of substrates from the ionic liquid [BMP][NTf2] alluded to in Sections 5.4 and 5.10.3 have been shown through Lewis Cell analysis of the [BMP][NTf2] system in comparison to toluene. Under conditions of fixed interfacial area between the two liquids mass transfer from [BMP][NTf2] to buffer was clearly superior to mass transfer from toluene to buffer.

The issues of substrate solubility, biocatalyst stability and mass transfer have been examined in this work. The influence of ionic liquids on enzyme activity appears to be the most critical followed by the inter-related effects of mass transfer substrate solubility. A strategy for the high throughput screening of ionic liquids for biocatalytic applications should centre upon these three factors and is described in Chapter 6.

6 A systematic approach to the high throughput screening of ionic liquids as co-solvents for biocatalysis

6.1 Introduction and aims

In the previous chapters both whole cell (Chapter 3) and isolated enzyme (Chapter 5) ketone reductions have been shown to be feasible in a range of ionic liquids. In both cases efficient bioconversions for a number of substrates and biocatalysts were achieved by evaluating a relatively small range of ionic liquids (Tables 3.1 and 5.2). A number of common process design considerations were identified including substrate solubility, biocatalyst stability and the relative rates of bioconversion and solute mass transfer in the case of water immiscible ionic liquids.

The success of the screening approach, and the present inability to relate ionic liquid structure or properties to bioconversion efficiency, suggests that for the industrial uptake of ionic liquids a systematic, high throughput approach to ionic liquid evaluation is required. Building upon the earlier experimental results, the aim of this chapter is to outline and exemplify a systematic approach to ionic liquid selection and how it can be implemented. The specific objectives of this chapter are to:

- Outline a systematic approach to ionic liquid evaluation for biocatalytic applications.
- Describe methods for automated solubility studies and establish the congruence of automated methods to manual.
- Describe the use of such automated platforms for biocatalyst and co-solvent screening.
- Describe larger scale methods for determining rate of reaction and conversion data.

6.2 A systematic approach to ionic liquid selection

The vast array of enzymes and co-solvents for potential biocatalytic application requires a robust and verifiable method for screening. Any screening method needs to be rapid, small scale and preferably automated to allow research groups to establish product formation rates, chirality, ideal co-solvent (if any), substrate solubility and biocatalyst kinetics at relatively low cost. The use of larger scale bio-reactor mimics would also be required to subsequently establish process parameters relating to phase mixing and mass transfer.

Based upon the earlier findings in this study Figure 6.1 illustrates a proposed hierarchical approach to the systematic evaluation of ionic liquids as co-solvents for biocatalysis. It also indicates the scales of operation and the nature of the equipment that could be used at each stage. While the emphasis is on ionic liquids the same elements may be applied to any organic co-solvents.

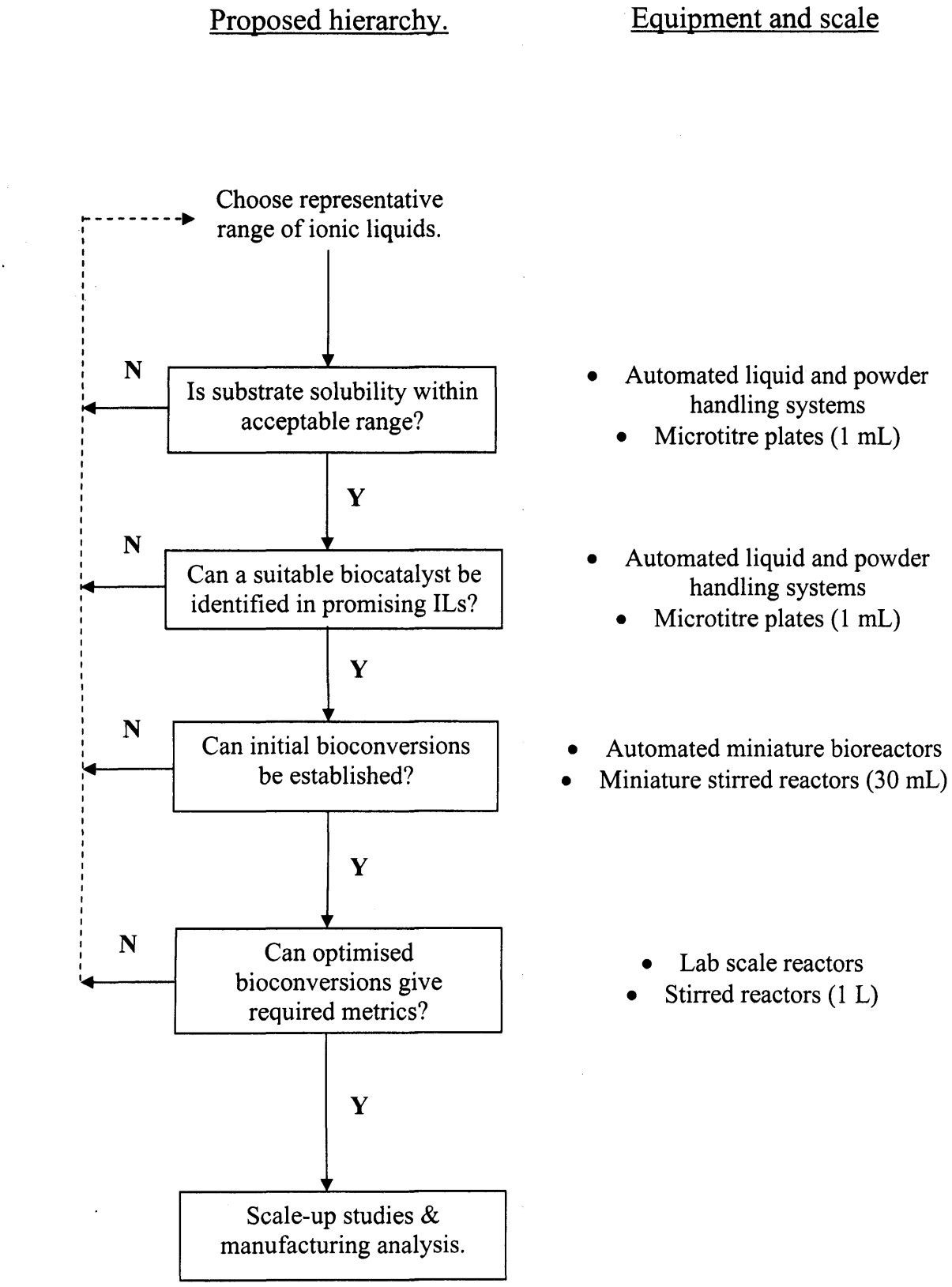


Figure 6.1 A hierarchical approach towards the systematic evaluation of ionic liquids as co-solvents for biocatalysis.

6.3 Automated solubility screen

The first experiments performed within the proposed framework relate to determination of the solubility of the substrate in a wide range of ionic liquids. To demonstrate this aspect of the approach a microwell platform capable of dispensing both solids and liquids was used to prepare microwell plates containing 250mg of the desired substrate 6-Br-β-tetralone (Figure 6.2). A microtitre plate containing 96 miniature glass vials (max volume 1.5 mL per well) was taken by the robotic arm and was placed on the integrated weighing platform. A shield then came up to surround the plate and protect it from any air disturbances during solids dispensing and the weight measurement. To fill each well the robotic arm then picked up a hopper from a defined position, prefilled with the solid substrate required, and added a defined amount to each vial. Solids could be dispensed to an accuracy of ±0.2 mg.

The powder filled block was then moved to the liquid handling station (Figure 6.3). Here, solvent was taken from 32 possible positions and added to the pre-dispensed solids in each well as required. A particular issue with the use of automated liquid handling systems with ionic liquids is the high viscosity of some pure ionic liquid phases. To overcome this problem specific "viscous tips" were used on the platform allowing more accurate aspiration. The aspirate and dispense speeds were then minimised to allow sufficient time for take up of the required volume of ionic liquid. However, even with these factors taken into account, the very viscous ionic liquids, such as [CABHEM][$MeSO_4$] were still not aspirated accurately.

(a)

(b)

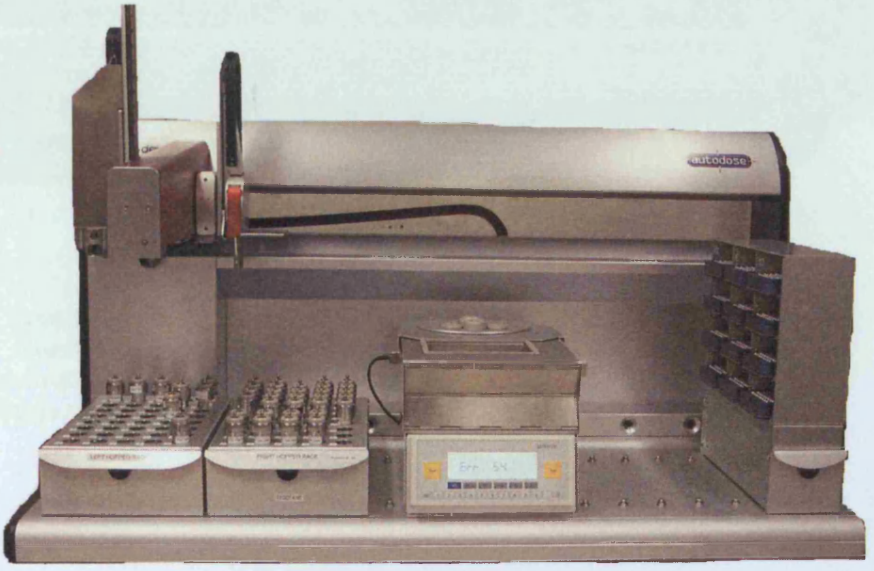


Figure 6.2 Elements used in the automated substrate solubility screen: (a) a 96 vial plate for automated solubility screening. (b) "Powdernium" solids dispensing system for addition of solid substrates. Images reproduced from http://www.symyx.com/page.php?id=74.

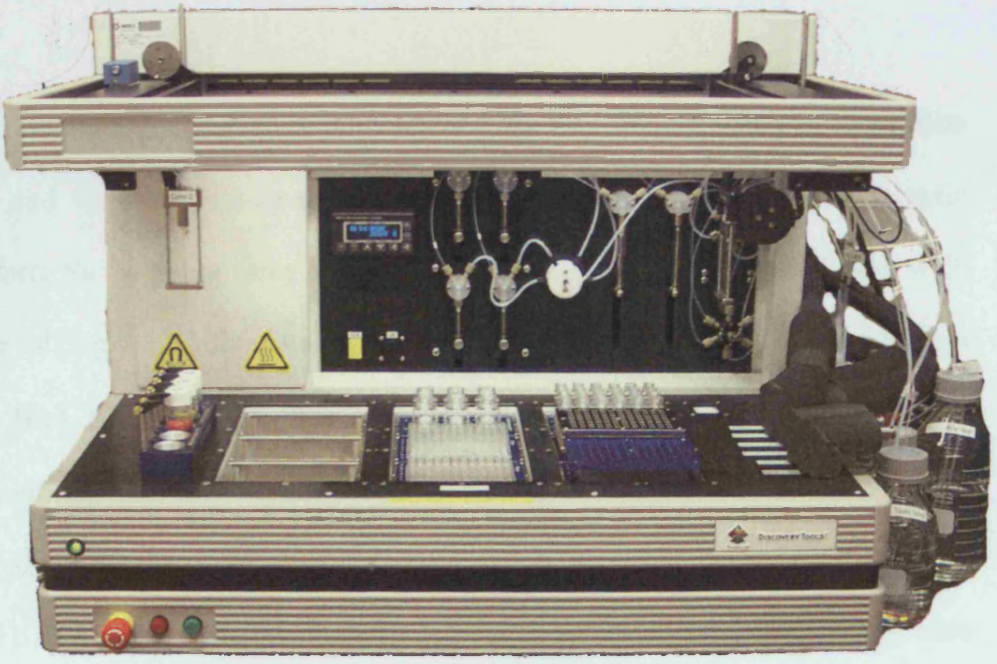


Figure 6.3 Symyx automated liquid handling platform for addition of liquid phases during the initial substrate solubility screen. The platform features 3 plate positions with independent heating and magnetic stirring controls, 4 tip arm, and 32 positions for additives. Image reproduced from http://www.symyx.com/page.php?id=70.

To fully overcome this problem, a "wait" step of 10 seconds was added while the tips were still submerged in the fluid to allow complete take up of the required ionic liquid volume. Use of glass vials rather than plastic multi-well plates allows for use of a full range of solvents as well as magnetic stirring ensuring good mixing of two phases. There is also the advantage that the vials are available for individual visual inspection which is not possible with rigid plates.

Once liquid dispensing was complete, the plate was covered using a rubber plate seal and the substrate-co-solvent mixture was left to stir on the magnetic platform for 24 hours using a 5 x 2 mm magnetic flea in the base of each well. After mixing the whole plate was centrifuged to take all the solids to the bottom and 50µl of the liquid was removed automatically and diluted in 950µl acetonitrile. This was subsequently diluted again 1:4 with acetonitrile before analysis of the dissolved substrate concentration by reverse phase HPLC (Section 2.8.1). The results obtained with the automated solubility screening system were compared to the manual solubility screen performed as described in Section 2.3.

The results shown in Figure 6.4 indicate a good agreement between automated and manual solubility studies. The three outlying points are identified as the ionic liquids [BMP][NTf2], [Bmim][BF_4] and AmmoEng^{TM} 120 all of which have relatively high viscosities. Further manipulation of the pipetting regime would be required to account for high viscosities as the automated system currently under predicts the substrate solubility.

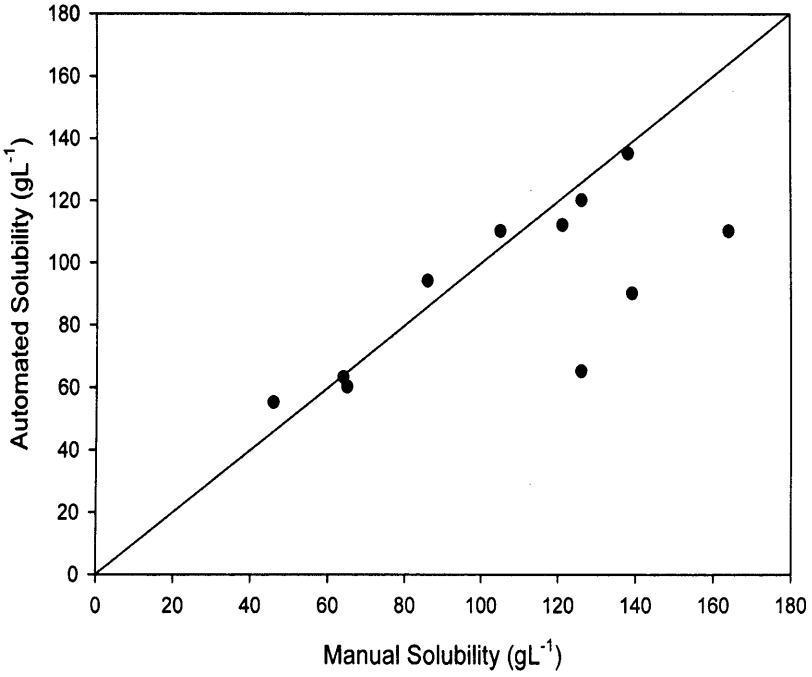


Figure 6.4 Parity plot between manual and automated solubility studies for the saturation solubility of 6-Br-β-tetralone in a range of ionic liquids and organic solvents. Solid line represents line of parity. Manual and automated solubility studies performed as described in Section 2.3 and 6.2 respectively. $R^2 = 0.953$.

6.4 Automated biocatalyst screen

The second element of the proposed framework relates to the identification of an appropriate biocatalyst, either whole cell or isolated enzyme, that is able to selectively transform the substrate of interest (Figure 6.1). This could be carried out using a similar robotic platform as was described in Section 6.3, and a possible method is described in Section 2.5. Product yield and enantiomeric excess can be determined for a library of biocatalysts in less than 24 hours (Table 5.1), and an appropriate biocatalyst identified.

6.5 Automated quantification of bioconversion kinetics

Having identified a suitable biocatalyst that acts on the substrate of interest in perhaps a small range of ionic liquids the next step of the framework is to gain more insight into the factors that influence bioconversion kinetics. As shown in Figure 6.1, these relate first to understanding the biocatalyst stability in the selected ionic liquids and later the relationship between the substrate mass transfer biocatalyst activity.

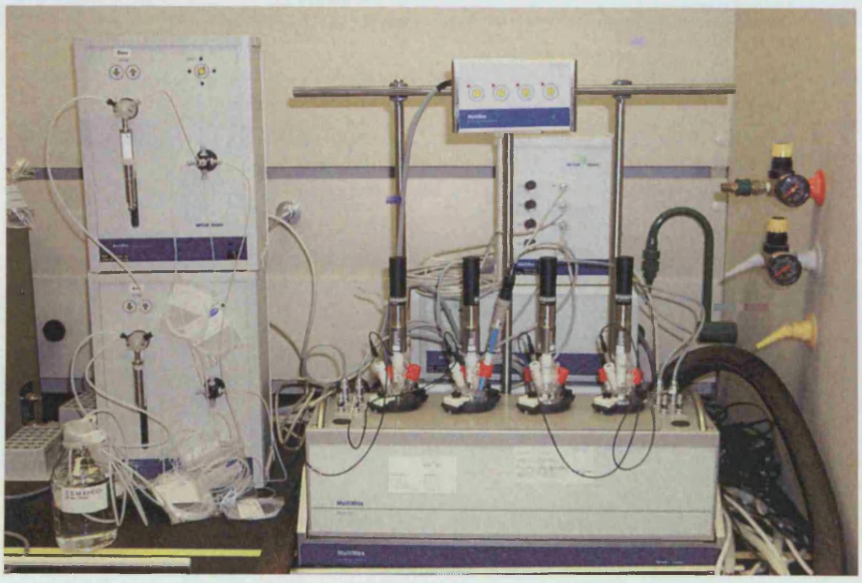
Analysis of the reaction kinetics of biocatalysts in the identified solvents was carried out in the Multimax™ miniature reactors (Figure 6.5). In this system, four reactors can be operated each with individual control of pH, temperature and rotational speed of the mixing shaft. The vessels have a liquid capacity of 50 mL with working volumes of 25-30 mL. Control of pH and temperature is dynamic via the pH and temperature probes visible in Figure 6.5(b). The associated

software monitors and records data for all the variables and this data is available via a graphical user interface (Figure 6.6) or via export to a Microsoft Excel format for ease of subsequent analysis.

In the case of isolated enzyme bioconversions, the GDH 103 co-factor recycling system used with both 6-Br-β-tetralone (Figure 5.1) and 4'-Br-2,2,2-trifluoroacetophenone (Figure 5.7) results in a decrease in pH of the aqueous phase due to the formation of gluconic acid from glucose. In this case, base addition to the reaction can be used to estimate on-line the initial rate of reaction and subsequent conversion.

The use of these automated miniature reactors is also likely to give a better indication of bioconversion kinetics in larger scale stirred reactors. In Chapters 3 and 4 whole cell bioconversions were routinely carried out in Erlenmeyer flasks mixed on a shaking incubator. The potential for much better mixing to eliminate any potential mass transfer limitations in these systems was demonstrated in the Multimax™ reactors (Figure 6.7). In the 20% v/v [Bmim][PF_6] system reported in Section 3.4.1 with the yeast *T. capitatum* MY1890 acting as the biocatalyst it was postulated that this system was mass transfer limited due to the linear increase in product concentration over time.

(a)



(b)

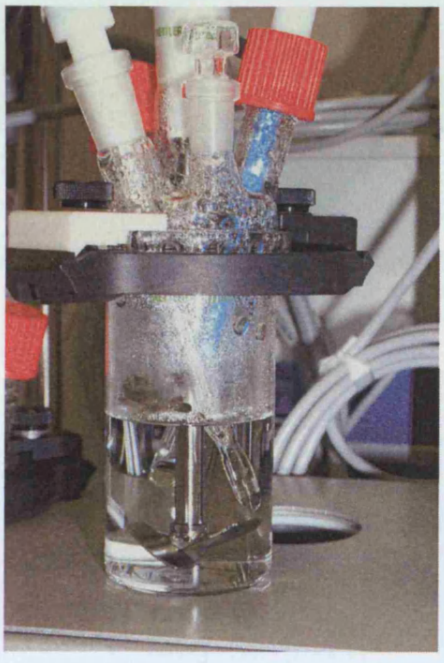


Figure 6.5 Automated miniature reactors for evaluation of bioconversion kinetics and related mass transfer studies: (a) Multimax™ miniature reactor system with 4 independent temperature control chambers and impeller rotational speeds. (b) detail of a Multimax™ reactor with pitched blade impeller (d_i = 24 mm, OD = 39 mm, ID = 35 mm, h = 74 mm) and pH and temperature probes.

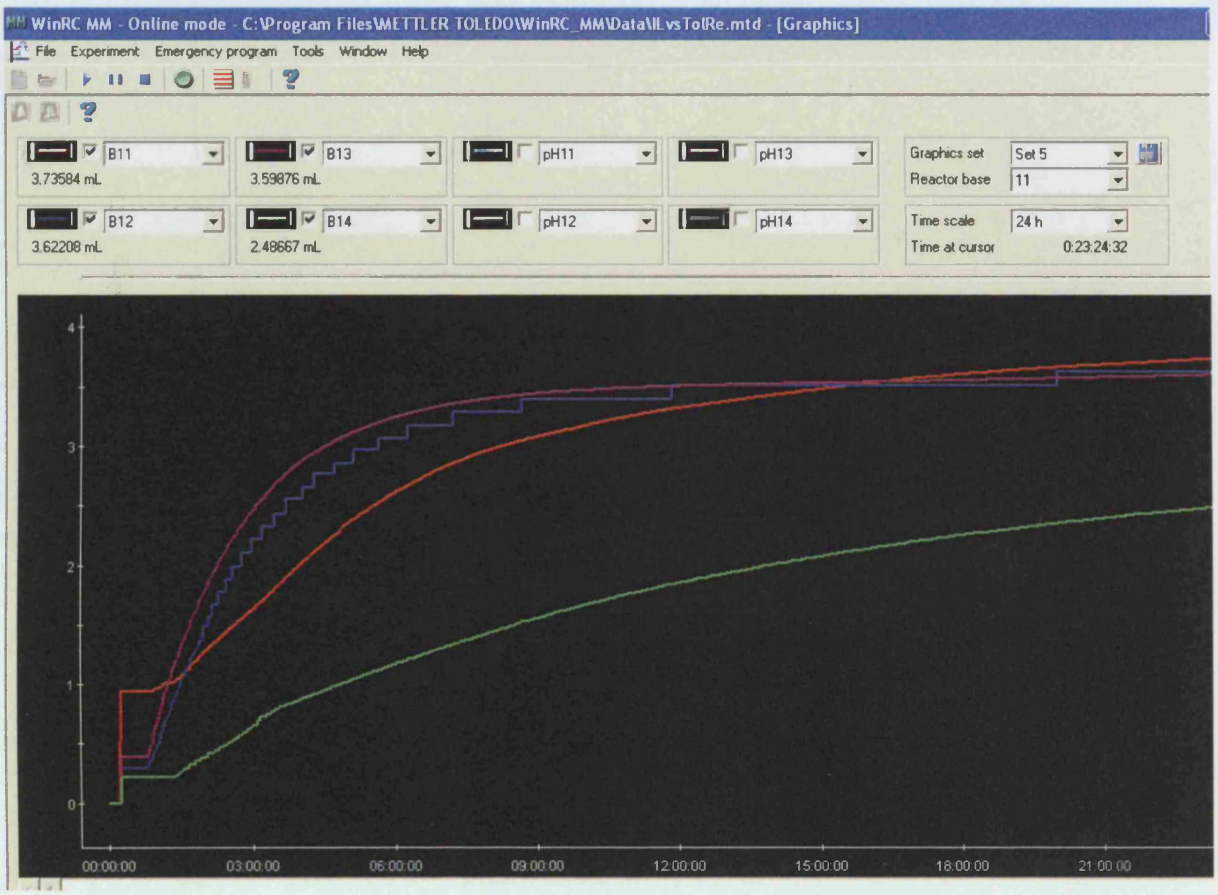


Figure 6.6 Example of online data collection from the Multimax™ system shown in Figure 6.5. The screen image shows base addition volumes to the four individual reactors. Temperature and pH can be similarly monitored for the full reaction course. Experiments were performed as described in Section 2.7.2.

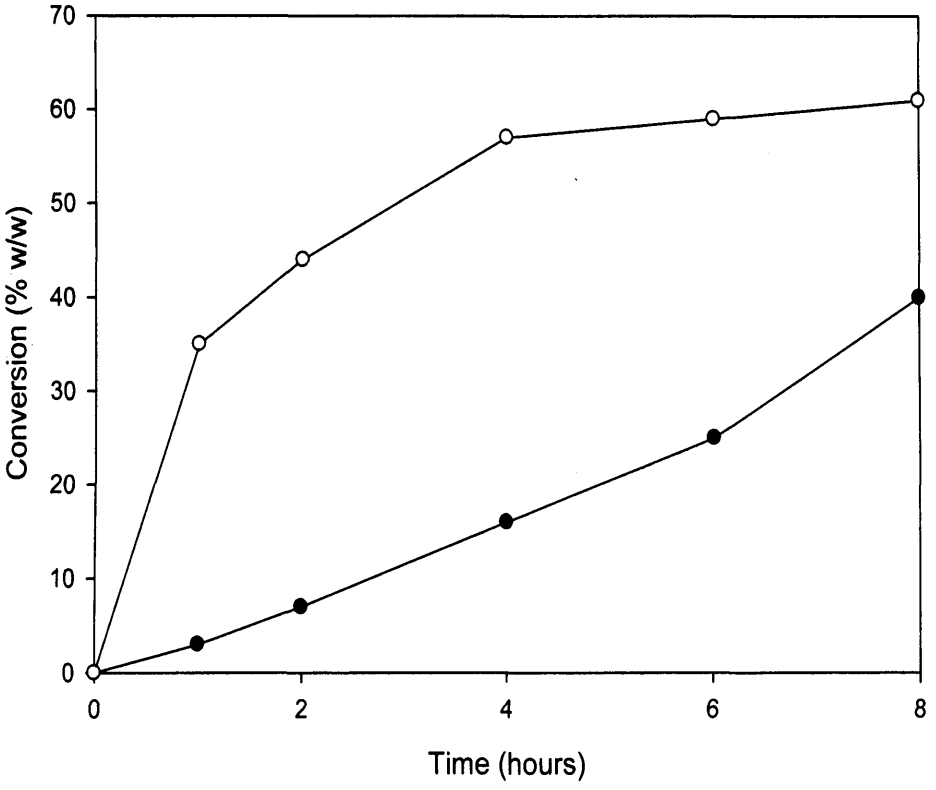


Figure 6.7 Conversion kinetics of 6-Br-β-tetralone reduction by the yeast *T. capitatum* MY1890 in the presence of 20% v/v [Bmim][PF_6] in: (•) 250ml Erlenmeyer flask cultured in an orbital shaker at 220 rpm and (○) Multimax™ miniature reactor operated at an impeller speed of 600 rpm. Cultivation was performed as described in Section 2.6 and bioconversion as described in Section 2.7.1.

As shown in Figure 6.7 the initial rate of reaction with mechanical agitation is around 7 fold greater than in the shaker flasks. However, conversion is minimal after approximately 4 hours indicating that the more intense mixing regime may have an adverse effect on the ability of the cells to perform the bioconversion. The mass averaged Reynold's number in this experiment was only 115 indicating a laminar regime, but this value comes about as a result of the much greater viscosity of the ionic liquid over water (0.352 Pas), and a higher density (1400 kgm^{-3}). However, as the biocatalyst resides only in the buffer phase, the relatively high rotational speed may have had an adverse effect on the biocatalyst stability. In summary, it has been shown that the use of a mini reactor system can be used to rapidly determine biotransformation kinetics with minimal human input while reducing any potential mass transfer limitations.

6.6 Analysis of biocatalyst stability

As described in Section 4.4.3 there is a strong relationship between biocatalyst stability in ionic liquids and the ultimate bioconversion performance. Consequently high throughput methods to evaluate whole cell biocatalyst stability are included as part of the framework (Figure 6.1) to help in the identification of a suitable biocatalyst. In Chapter 4 the utility of flow cytometry for the study of biocatalyst stability was demonstrated and it was mentioned that microwell plate based flow cytometry devices are now available.

In the case of isolated enzyme systems stability can already be monitored using a high throughput plate based assay via spectrometry as described in Section 2.8.4.

This system monitors the accumulation or consumption of the co factor NADH at a wavelength of 340nm. By comparing the rate of change in intensity to a standard concentration of enzyme an effective enzyme concentration can be determined over time during exposure to different ionic liquids.

For whole cells, however, the state of the biocatalyst has been measured specifically via multi parameter flow cytometry as described in Chapter 4. Recently, Guava™ Technologies have commercialised the EasyCyte flow cytometer system specifically for use with multi well plates as shown in Figure 6.8. Experience from our laboratory has shown that multiparameter analysis can be carried out rapidly (less than a minute per sample) on multiple biocatalyst samples using this device hence no specific studies were required here to confirm this element of the proposed framework.

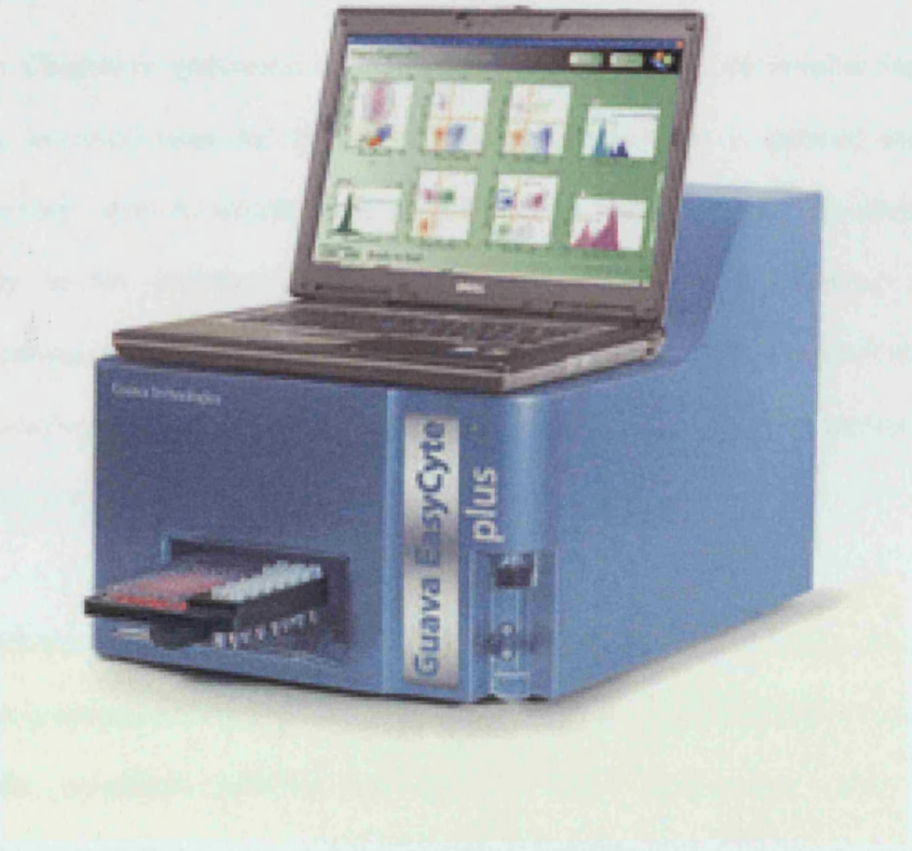


Figure 6.8 Illustration of the Guava™ Technologies Easy Cyte microwell based flow cytometry studies. The device features forward scatter, side scatter and 3 wavelength detectors for fluorochromes and can be readily integrated with any standard liquid handling robot. (http://www.guavatechnologies.com/main/products/easycyte-new.cfm).

6.7 Summary

In this Chapter a systematic approach to the identification of suitable ionic liquids as co-solvents for biocatalysis is described. This is centred upon determining aqueous solubility of substrate from the co-solvent, biocatalyst stability in the presence of co-solvent and substrate, and screening for biotransformation kinetics. Each of these processes has been shown to be readily automatable in multi well formats or miniature stirred reactors to allow screening of multiple conditions in parallel.

The saturation solubility of substrates into ionic liquids and solvents can be accurately ascertained through an automated process of weighed addition of solid substrate, co-solvent addition and agitation under temperature controlled conditions. Parameters such as the aspiration and dispensing speed of the liquid handling platform can be manipulated to take into account the particular properties of viscous materials like ionic liquids in order to improve measurement accuracy.

Biocatalyst and solvent screening is currently a standard technology for organic solvents in the case of whole cells (Stahl *et al*, 1997) and isolated enzymes (Pollard *et al*, 2006*b*; Truppo *et al*, 2006), and the inclusion of ionic liquids into such screening approaches provides a valuable addition to the range of process options that can be considered. Through the manipulation of liquid handling conditions described in Section 6.3 it is possible to accurately and reproducibly

dispense ionic liquids for analysis of bioconversion kinetics in a multiwell format.

Having identified biocatalysts and co-solvents further investigation of the biocatalyst stability and bioconversion kinetics must be carried out in larger scale devices of a similar geometry to pilot and manufacturing scale vessels. Here the biocatalyst can be exposed to a hydrodynamic environment more typical of that like to be present in a large scale system. The Multimax™ miniature bioreactor system with temperature and pH control and online monitoring has been shown to be ideal for this kind of analysis in the case of isolated enzymes (Section 5.10.3). Other products are now coming on to market for whole cell work which would allow analysis of both cell growth and bioconversion kinetics in a single vessel (Gill *et al*, 2007).

The work presented in this chapter have thus built upon the experimental studies described in Chapters 3, 4 and 5. A number of key engineering parameters for ionic liquid selection were identified and a hierarchical approach to ionic liquid process design was proposed (Figure 6.1). It has also been shown that the experiments required at each stage of ionic liquid evaluation can be readily automated allowing for high throughput, parallel experimentation.

7. General discussion and commercial issues

7.1 General discussion

The use of room temperature ionic liquids as co-solvents for biocatalytic redox reactions, in both whole cell and isolated enzyme conversions, has been successfully demonstrated in this work The main factors concerning their implementation were the solubility of the substrate in the co-solvent mixture, the stability of the biocatalyst in the presence of the co-solvent and the mass transfer efficiency of the transfer of substrate and product to and from the aqueous phase in the case of two-phase forming co-solvents.

One of the primary advantages of ionic liquids cited in the literature is that they are good solvents capable of dissolving large quantities of substrate (Cull *et al*, 2000; Roberts and Lye 2002; Mateus *et al*, 2003; Roberts *et al*, 2004). In this work it has been found that this is not always the case for certain types of substrate. Table 5.2 shows that of the ionic liquids screened some have significantly lower solubility of 6-Br-β-tetralone than common organic solvents such as toluene, THF or DMSO. However, when considering the supply of substrate to the environment of the biocatalyst, which resides in the aqueous phase, ionic liquids are generally superior to the organic solvents mentioned in Table 5.2. This is attributed to the higher polarity of the ionic liquids, with polarities generally in the range 0.7-0.8 relative to water, compared to solvents such as toluene with polarity of ~0.1 relative to water. In the case 4'-Br-2,2,2-trifluoroacetophenone, the substrate solubility in buffer alone was already quite

high (~6 gL^{-1}) but use of ionic liquids such as AmmoEng™ 110 and EcoEng™ 1111P doubled the substrate solubility in the co-solvent mixture at equilibrium (Table 5.5).

The stability of the biocatalyst in the presence of the co-solvent under consideration is an important consideration in co-solvent selection. In the case of *T. capitatum* MY1890 stability of the biocatalyst was markedly affected by the presence of any co-solvent (Table 3.2). Two to three log reductions in viable cell numbers were recorded within eight hours by colony forming unit counts (Table 3.2). The effect of ionic liquid co-solvents, in general, was intermediate to that observed in the presence of common organic solvents such as ethanol or toluene compared to media only (Figure 3.3). There was no marked difference observed between single phase forming ionic liquids and those immiscible with water forming two phases. This is contrary to general observations for two phase systems where partitioning of the solvent away from the biocatalyst in the aqueous phase promotes stability (Cao *et al*, 2004). In the case of *R. erythropolis* MA7213 the effect of co-solvents was generally less pronounced as expected for Gram positive bacteria (Harrop *et al*, 1992). Some single phase forming ionic liquids such as [Emim][TOS] and [Bmim][BF_4] exhibited biocatalyst stabilities superior to the cells residing in buffer only (Table 3.3). Both of the water immiscible ionic liquids tested, [Bmim][PF_6] and [Oc_3MeN][NTf2] similarly conferred some degree of greater stability on the gram positive biocatalyst compared to buffer only. In the case of immiscible co-solvents the apparent stabilising effect can be attributed in part to the removal of all soluble contaminants in the ionic liquid through washing prior to use (Section 2.2). In the

case of miscible ionic liquids the mechanism of stability conferred by the co-solvent mixture is currently unknown.

This quantification of whole cell biocatalyst stability based on colony forming unit counts relies upon the ability of cells to divide and form colonies on agar as a sign of their viability (Nebe-von-Caron *et al*, 1998). Although some degree of congruence was shown between the number of viable cells determined by this method and bioconversion kinetics, the speed and rate of bioconversion observed suggests that despite the apparent stress the cells are exposed to, their ability to carry out the bioconversion does not appear to have been affected (Section 3.3.2). Other work (Cruz *et al*, 2004) in whole cell bioconversions has shown the efficacy of whole cell biocatalysts at carrying out selected bioconversions after being removed from growth media, washed and resuspended. This indicates that viability, as measured through an ability to divide is not required for effective bioconversion, but an active cellular metabolism is.

The use of multi parameter flow cytometry described in this work facilitates discrimination of the effect of co-solvents on whole cell biocatalysts (Chapter 4). As described in Section 1.8, cells undergo a period of stress, known as depolarisation, prior to cell death where the cellular metabolic pathways begin to break down. After ATP breakdown has ceased, the presence and efficacy of membrane bound proteins does not necessarily cease, indicating a possible mechanism for the continued bioconversions observed. The parity between viable cell counts as determined through colony forming unit counts and flow cytometric determination of live polarised cell populations (Figure 4.4) indicates

the depolarised cells, which form a significant proportion of the cell number as determined through flow cytometry analysis could account for the apparent disparity between colony forming unit numbers and bioconversion kinetics.

In the case of isolated enzyme bioreductions the use of an immiscible ionic liquid, [BMP][NTf2], has been found to lead to a dramatic improvement in reaction performance for both of the bioconversions studied in this work (Figures 5.2 and 5.8). This increase in performance is a combination of increased half-life of both ADH RE and the GDH 103 co-factor recycling enzyme (Table 5.3) and other effects, most notably mass transfer (Sections 5.5 and 5.12). The often greatly increased enzyme half-life in the presence of hydrophobic co-solvents is attributed to a number of possible mechanisms. Hydrophilic co-solvents have been found to strip enzymes of "internally bound" water and soluble components of solvents interact electrostatically to adversely affect enzyme activity. In contrast, the action of hydrophobic co-solvents such as [BMP][NTf2] seem to promote enzyme stability (Zhao, 2005). A more compact enzyme conformation resulting from changes of α-helix structures to β-sheet within the protein has also been observed with hydrophobic ionic liquids (De Diego *et al*, 2005) and could act to protect the enzyme within a co-solvent system. For the reduction of 4'-Br-2,2,2-trifluoroacetphenone [BMP][NTf2] acted to decrease the inhibitory effect of the substrate on the ADH RE. Potential changes in the redox environment within a co-solvent solution may also have an effect on the dynamics of the co-factor binding and transfer within the system (Ichinose *et al*, 2005; Edegger *et al*, 2006). This can subsequently affect the binding and transfer of substrate resulting in the kinds of changes in initial rate of reaction observed in this work.

In a number of cases it has been shown that the use of ionic liquids can improve the mass transfer of substrates from the co-solvent phase to the aqueous phase in heterogeneous reaction systems. In Section 5.12 it was shown that despite a significant difference in Reynolds number the mass transfer of 6-Br-β-tetralone from the ionic liquid [BMP][NTf2] into the aqueous buffer phase was much more rapid than the transfer of the same substrate from the organic solvent toluene into the aqueous buffer phase given the same volume fractions and interfacial area (Figure 5.11). Coupled with the increased stability of the isolated enzyme biocatalyst observed in Section 5.4 the superior initial rate of reaction observed and more rapid overall conversion can be accounted for. A similar pattern was observed in the case of the 4'-2,2,2-trifluoroacetophenone reduction and it is highly likely that the mass transfer of this substrate is generally superior from the ionic liquid into the aqueous phase.

These two parameters: biocatalyst stability and mass transfer coefficients appear to be the most significant in determining successful application of ionic liquids to a process. However, solubility of the substrate is also an important consideration as illustrated by the AmmoEng™ 102 data for the two isolated enzyme reductions. Despite a poor enzyme stability, with ADH RE half life measured at just 12 hours (Table 5.3), the 6-Br-β-tetralone bioreduction proceeds to 100% w/w conversion at an initial rate of 6.5 $g(prod)L^{-1}hr^{-1}$. With such a low enzyme half life this would not be expected but the solubility of 6-Br-β-tetralone in the co-solvent mixture is enhanced to ~6 gL^{-1} in the presence of 10% v/v

AmmoEng™ 102. This higher initial substrate concentration appears to drive the reaction to completion at a fast initial rate.

Based on these experimental studies the parameters for successful selection of ionic liquid co-solvents have been established. In Chapter 6 a systematic framework for the identification and selection of biocompatible ionic liquids has been suggested. Automation allows for rapid, parallel screening of parameters such as solubility and biocatalyst stability facilitating co-solvent identification (Lye *et al*, 2003). It is critical that this framework includes analysis of reactions and reaction conditions as other advantages of ionic liquids such as superior mass transfer cannot be readily directly identified through a high throughput screening process. However, a rapid method for identifying an appropriate biocatalyst, stability of that biocatalyst and substrate solubilities in a range of co-solvents, and subsequent analysis of rates of reaction and conversion in larger scale miniature reactors is a feasible method for process development. The automation of all the elements of the proposed framework has been successfully demonstrated. The successful use of small libraries of commercially available ionic liquids in this work and others (Roberts *et al*, 2004; Lozano *et al*, 2006; Zhang *et al*, 2006) overcomes the significant hurdle of the vast numbers of potential ionic liquids and their tenability which is antithetic to rapid process development.

7.2 Regulatory issues[1]

The robust, tightly controlled regulatory environment of the pharmaceutical sector is a feature of medicine development that must be considered from the very beginning of process development. There are a number of potential issues surrounding the use of ionic liquids for pharmaceutical drug development that must be addressed before they can become a common feature of industrial bioconversion processes.

The issues of residual solvents in drug preparations were addressed by the Centers for Drug Evaluation and Research (CDER) and for Biologics Evaluation and Research (CBER) of the U.S. Food and Drug Administration (FDA) in 1997. For the purposes of safety assessment the CDER and CBER have classified solvents into three classes:

- Class 1: encompassing solvents known or suspected to be human carcinogens or environmentally hazardous.
- Class 2: solvents that are non genotoxic but possibly cause other irreversible toxicity such as neurotoxicity or teratogenecity.
- Class 3: solvents with low potential human toxicity that are known to have a permitted daily exposure (PDE) limit of over 50 mg.

Solvents within class 1 are to be totally avoided, class 2 solvents are permitted to be used in a limited fashion up to their PDE value with analysis of their impact

[1] The inclusion of this section relates to the University of London EngD requirement for consideration of the validation implications of the research work performed.

on health over long term exposure times. Class 3 solvents can be used such that their daily intake would be 50 mg or less without justification and above that limit up to their PDE with long term exposure studies.

The safety implications of ionic liquids, particularly in humans, is not well understood at the moment. Some work has taken place into aquatic models (Zhao *et al*, 2007) which shows that many of the ionic liquids currently in use in ionic liquid research are highly toxic: the ionic liquid [Bmim][PF_6] has an LC_{50} value of 19.9 and [Bmim][BF_4] an LC_{50} value of 10.7 to the aquatic *Daphnia* genus as well as poor biodegradability (Wells and Coombe, 2006). Compared to methanol, the toxicity of the ionic liquids tested by Wells and Coombe is of the order of 10^4 to 10^6 greater. This indicates that the safety of ionic liquids needs to be of primary concern to the manufacturers and pharmaceutical end users. In this work, it was shown that the product of interest could be very effectively extracted from the ionic liquid phase using organic solvents but the residual concentration of the co-solvent in the product preparation could not be determined. Work in this area would also be valuable as it is only residual concentrations of solvents in the final formulated product that is covered by the FDA regulations mentioned earlier. The toluene used for isolation of 6-Br-β-tetralol in Chapter 5 is a class 2 solvent within the FDA classification system with a PDE of 8.9 mg day^{-1}.

A further potential regulatory issue associated with ionic liquids is their safe disposal. As non volatile ionic salts, ionic liquids are generally very stable over wide temperature ranges and they have been shown to be poorly biodegradable

in their complete form. Development of safe methods for effective disposal of ionic liquids are thus of paramount importance at the present time. Recent work in this area by Li and co-workers (Li *et al*, 2007) has shown possible methods and mechanisms for the oxidative degradation of imidazolium ionic liquids to inert components under mild conditions (50°C and 2.5mM anion concentration). Further work is required for other salts to determine generic methods to overcome this regulatory barrier to more widespread ionic liquid usage.

Although ionic liquid manufacture is becoming more widespread, and more companies are becoming active in ionic liquid manufacture and distribution, a consistent supply of good quality ionic liquids is required for FDA approval. Issues such as soluble contaminant concentrations and unreacted halide prevalence (as found in practice) between batches of ionic liquids must be addressed before industrial uses of these valuable solvents can increase.

The primary regulatory barrier to the use of ionic liquids in pharmaceutical processes at this time is the lack of safety data for a wide enough variety of ionic liquids. The lack of work on suitable models for extrapolation of data to human models is also a major concern. The limited data present in the literature appears to show that the toxicity of ionic liquids is such that they would be under Class 1 of the CDER solvent classification system. Effective means of purifying products such that no traces of ionic liquid remain would thus be required to overcome this regulatory constraint. Analytical methods such as LC-MS would be required to accurately quantify ionic liquid content of product extracts. Long term supply

of high quality, consistent batches of ionic liquids from manufacturers would also be required to ensure consistency of processes over drug product lifetimes.

7.3 Bioprocess management[2]

The development of new drugs is broadly a well defined process encompassing drug discovery and basic research, preclinical testing, clinical testing, regulatory review and approval and post-market testing. The rate of attrition of this process is well attested and it has recently (2005) been suggested that of 10,000 compounds screened through basic research only one will be approved as a pharmaceutical drug some 16 years later. Faster process development and higher yields of product from these processes is therefore a paramount concern to determine the potential of a drug candidate as soon as possible (http://www.merck.com/).

The work on the bioreduction of 4'-Br-2,2,2-trifluoroacetophenone (Section 5.10) clearly showed an application of ionic liquids that led to a previously infeasible process becoming potentially industrially viable. However, there are further considerations to be made with respect to ionic liquids in the pharmaceutical environment before they are taken up as a standard technology. Some of these considerations are related to the regulatory approval of processes that employ ionic liquids and were discussed in the Section 7.2. There are other considerations to be made on the basis of effective pharmaceutical management.

[2]The inclusion of this section relates to the University of London EngD requirement for consideration of the bioprocess management implications of the research work performed.

The primary concern here is the relatively much greater cost of ionic liquids compared to organic solvents. The ionic liquid [BMP][NTf2] currently sells for £370 per kilogram. In contrast 1 litre of high purity toluene retails for £15. However, the financial considerations are based not only on the cost of purchasing ionic liquids but also of their disposal. As discussed in Section 7.2 a chemical degradation process is required to safely dispose of ionic liquids significantly increasing the costs of use. This is because either a second plant will be required on the manufacturing site to carry out the degradation process, or the disposal will need to be contracted out to a second company to dispose of the ionic liquids safely.

The greatest advantages with ionic liquids have been observed in this work with regard to isolated enzyme processes. These are generally preferred processes due to the ability to rapidly develop a process for effective product synthesis. The major issue with isolated enzyme redox syntheses, however, is the requirement for co-factor, as has been discussed in Section 1.2. In this work, this issue has been overcome through the use of a coupled enzyme system converting an inert second substrate to regenerate co-factor (Figure 5.1). This has significant implications for cost as enzyme preparations are generally the most expensive component in the system. Recently, isolated enzyme process development requiring co-factor has employed the second recycling system postulated by Kroutil and co-workers (Kroutil *et al*, 2004). This process employs a single enzyme that recycles a coupled substrate, often isopropyl alcohol in which the substrate can be suspended. This is shown in Figure 7.1. The effect of the isopropyl alcohol on the ionic liquid present and the stabilisation or improved

mass transfer effects observed and discussed in Chapter 5 would be unknown. However, through this kind of process improvement significant cost savings can be made over a system employing two enzymes.

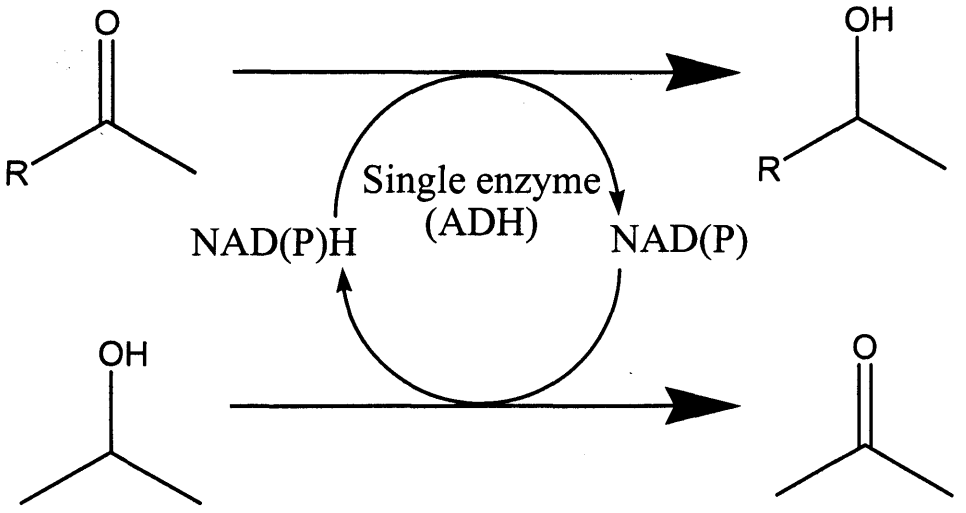


Figure 7.1 The coupled substrate recycling of NAD(P)H cofactor during the enzymatic reduction of a ketone to alcohol. (Kroutil *et al*, 2004).

Long term supply and stability of the ionic liquid co-solvent discussed in the previous section would also be a concern here. The process would be reliant on consistent batches of ionic liquids that are supplied through a robust chain. As yet, there are too few examples of ionic liquids being made and distributed at large scale to assuage the concern over security of the supply chain. Long term stability studies of the ionic liquids are required to determine the size of batches that can be supplied in single loads and kept for use within the drug manufacturing company. The ionic liquid [Bmim][PF_6] was reported to be degrading to hydrogen fluoride (Swatloski, 2003) through a hydrolysis reaction

which is highly toxic and corrosive indicating the need for robust testing of ionic liquid products before widespread use.

Finally, the issue of the high cost of ionic liquids will be resolved as more and more groups make use of their unique properties and processing advantages such as those outlined in this work. The only major industrial process involving ionic liquids to date is the BASF process involving scavenging of acid at the end of the process for synthesis of alkoxyphenylphosphine. The ionic liquid that forms at the end of this process is an immiscible dense liquid also facilitating product recovery (Seddon, 2003). As ionic liquids attract more interest and processing advantages become more well known the potential for ionic liquids to be used as a cost effective process component may be realised.

8 Conclusions and future work

8.1 Conclusions

In this work the successful use of ionic liquids as co-solvents in biocatalytic redox reactions has been demonstrated. The bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the whole cell strains *T. capitatum* MY1890 and *R. erythropolis* MA7213 in the presence of 20% v/v [Emim][TOS] was shown to be either comparable in the case of *T. capitatum* MY1890 (Figures 3.2(a) and 3.4(a)) or superior in the case of *R. erythropolis* MA7213 (Figures 3.2(b) and 3.5) to the base case control reaction involving 10% v/v ethanol as co-solvent. The decrease in cell viability of both types of cell upon exposure to ionic liquids (Figure 3.3) was found to be intermediate to that determined for cells residing purely in fermentation media and cells residing in a two-phase mixture of media and organic solvent (toluene). For *T. capitatum* MY1890 bioconversions, the water miscible hydrophilic ionic liquid [Emim][TOS] gave a reaction profile comparable to that observed in the previously studied water-ethanol (10% v/v) system (Figure 3.4(a)), in terms of overall rate of reaction ($0.2g(prod)L^{-1}hr^{-1}$) and conversion (100% w/w). Of the hydrophobic ionic liquids evaluated [Oc_3MeN][NTf2] gave the best conversion of 60%, but at a much reduced rate suggesting solute mass transfer from the ionic liquid phase was rate limiting (Figure 3.4(b)). For bioconversions carried out with *R. erythropolis* MA7213 employing 20% v/v [Emim][TOS] as a co-solvent the conversion yield doubled and a four-fold increase in initial rate was found (Figure 3.5) compared to the standard ethanol co-solvent (Figure 3.2(b)). This was attributed to improved cell

viability and reduced aggregation of the *R. erythropolis* MA7213 compared to *T. capitatum* MY1890 (Figure 3.6).

Multi parameter flow cytometry was used for the first time to determine the biocompatibility of ionic liquids with whole cell biocatalysts in a rapid manner (Chapter 4). It was shown that the traditional method of counting viable cell colonies on agar plates was equivalent to the polarised live cell counts obtained through flow cytometry (Figure 4.4). As suspected, the mechanism of toxicity of the ionic liquids appears to be to disrupt the cellular metabolism leading to cell death (Figure 4.8(b)). Flow cytometry was successfully used to identify the most biocompatible ionic liquid for the gram negative bacterium *E. coli* TOP10 pQR239 (Figure 4.7), and this ionic liquid, [Emim][TOS] was successfully used as a co-solvent to double conversion of the substrate 1-indanone into 3,4-dihydrocoumarin (Figure 4.9 and Table 4.3).

The work described in Chapter 5 is the first example of the isolated enzyme mediated reduction of a ketone with *in-situ* recycling of the co-factor in the presence of ionic liquids at industrially relevant substrate concentrations. The enantioselective reduction of 4'-Br-2,2,2-trifluoroacetophenone to (*R*)-4'-Br-2,2,2-trifluoroacetophenyl alcohol was limited to a maximum alcohol production of 10 gL^{-1} using traditional organic solvents whereas use of the ionic liquid [BMP][NTf2] led to complete conversion of 50 gL^{-1} substrate at a high rate and with excellent enantioselectivity (Figure 5.8). The reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol was also improved through use of ionic liquids (Figure 5.3). Product recovery and isolation was demonstrated with yields of 85% w/w in

the case of (*R*)-4'-Br-trifluoroacetophenyl alcohol (Section 5.11.6) and 88% w/w in the case of (*S*)-6-Br-β-tetralol (Section 5.10) with product ee >99% in favour of the desired enantiomer in both cases. The stability of the enzymes employed was shown to be increased through the presence of ionic liquid with half lives of the two enzymes employed increased to 266 hours in the case of ADH RE and >300 hours in the case of GDH 103 compared to 78 hours and 128 hours respectively in the presence of buffer alone (Table 5.3).

Finally, based on the preceding experimental results it is proposed that substrate solubility in the co-solvent mixture, biocatalyst stability in the presence of the co-solvent and mass transfer efficiency of the substrate from the co-solvent phase to the aqueous phase in the case of immiscible co-solvents are the key design features. A conceptual framework for the identification of ionic liquids as co-solvents for biocatalytic conversions has been proposed and the potential for automation has been shown (Figure 6.1). Design of a bioconversion process would thus be based on the identification of the biocatalyst, solubility of the substrate in a range of solvents and co-solvent mixtures, stability of the biocatalyst in the presence of the substrate in those co-solvent mixtures and the subsequent screening of reaction conditions. Such a system has been implemented at Merck & Co. Inc. and is currently in use.

8.2 Future Work

A number of potential areas for further investigation have been identified throughout the course of this work. Based upon the experimental results obtained here, the immediate follow-up studies should address the following points.

- The whole cell biocatalysts investigated in Chapters 3 and 4 were found to be stabilised by some ionic liquids as well- particularly those that form two phases. Further investigation should focus on whether this stability is a result of changes at a molecular level or whether the cells are simply being protected from phase interface effects.

- The effects observed in Chapter 5 of improved mass transfer coefficients from the ionic liquid into aqueous phase should be examined further to find whether this is a generic effect of ionic liquids. It would also be interesting to attempt to determine a molecular basis for the observed effects.

- The improved enzyme stability observed in Section 5.4 should also be examined further. It was postulated in Chapter 7 that the presence of ionic liquid prompts changes in the enzyme secondary structure from α-helix to β-sheet motifs and vice versa. This phenomenon could be examined through the use of circular dichroic spectrometry to determine if the stability is indeed conferred through structural changes within the enzyme molecule.

- A conceptual approach has been developed for selection of ionic liquids and demonstrated on a small scale in the case of the 1-indanone bioconversion covered in Chapter 4. More in-depth study of the factors identified for co-solvent selection should be carried out and a Design of Experiments matrix constructed (Islam et al, 2007) to facilitate rapid co-solvent selection.

Based upon the wider considerations of the use of ionic liquids in bioconversion processes, especially in an industrial context, the following points could be addressed.

- The thermal stability of ionic liquids and the stability of enzyme and whole cell biocatalysts have not been examined in any depth in this work. Future work could explore the effects of higher temperatures on rates of reaction and biocatalyst stability for a range of biocatalysts and substrates.

- In Section 7.2 the regulatory environment for solvent usage in pharmaceutical development was discussed. Quantification of residual ionic liquid concentrations within isolated product preparations should be investigated through the use of NMR and mass spectrometry.

- In view of the current high costs of both the ionic liquids and isolated enzyme preparations further study of the potential of ionic liquid re-use should be investigated to determine the number of times they can be recycled. Similarly, since the isolated enzymes investigated in this work have been shown to have improved stability in the presence of certain ionic

liquids the potential for the enzyme preparations to be re-used should be investigated. This could lead to a more comprehensive economic evaluation of the industrial use of ionic liquids.

- Whilst literature data and the results obtained here show that suitable ionic liquids for a particular bioconversion can be identified by screening a small range of ionic liquids, in the longer term it would be useful to examine and correlate the physico-chemical features of ionic liquids to both substrate solubility and biocatalyst stability.

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Appendix A: Reverse phase HPLC analysis

A.1 HPLC analysis of the bioreduction of 6-Br-β-tetralone to 6-Br-β-tetralol

A.1.1 Calibration curves

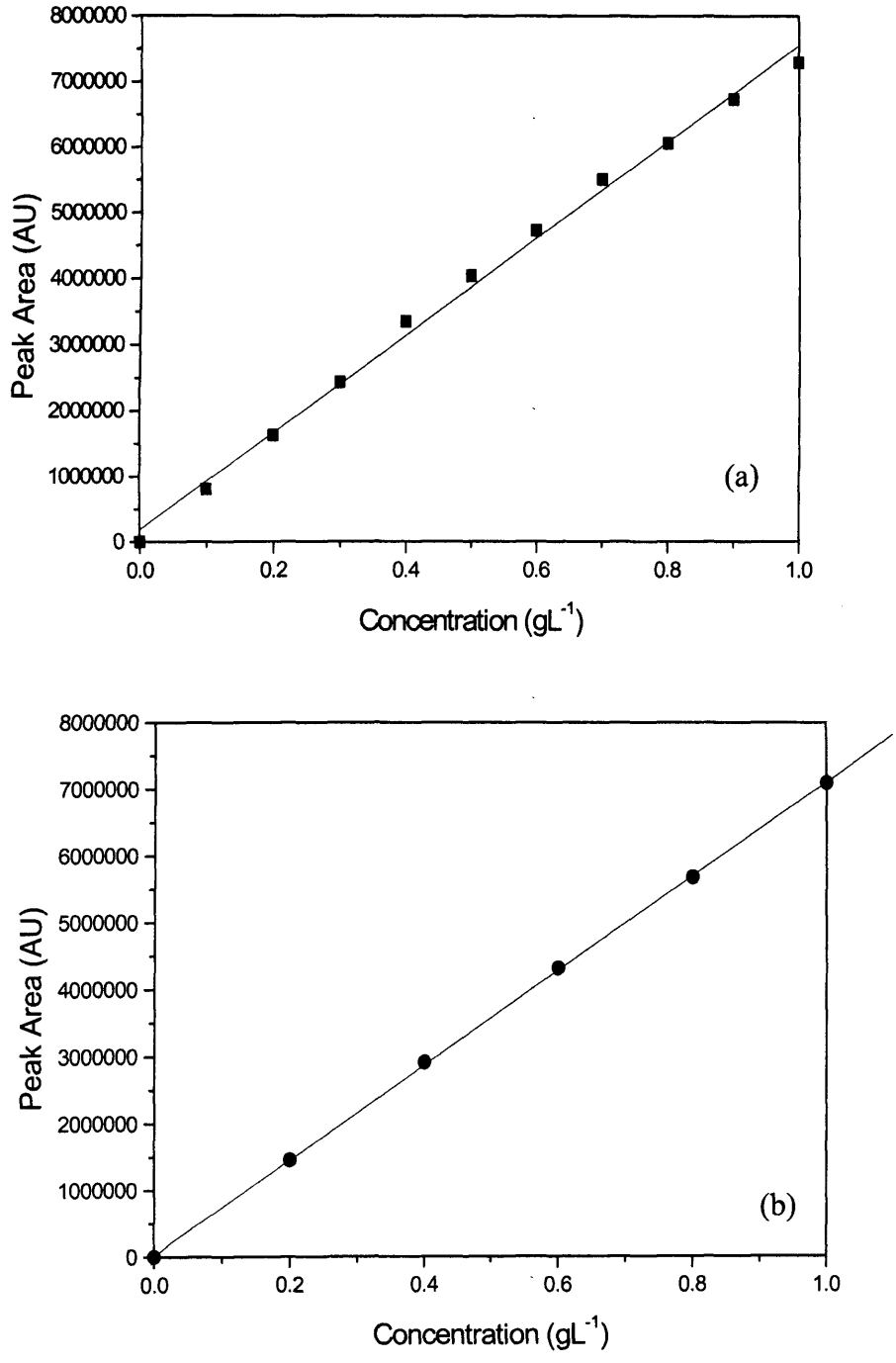

Figure A.1 HPLC calibration curves for (a) 6-Br-β-tetralone and (b) 6-Br-β-tetralol. Samples were analysed as described in Section 2.8.1.

A.1.2 Sample chromatogram

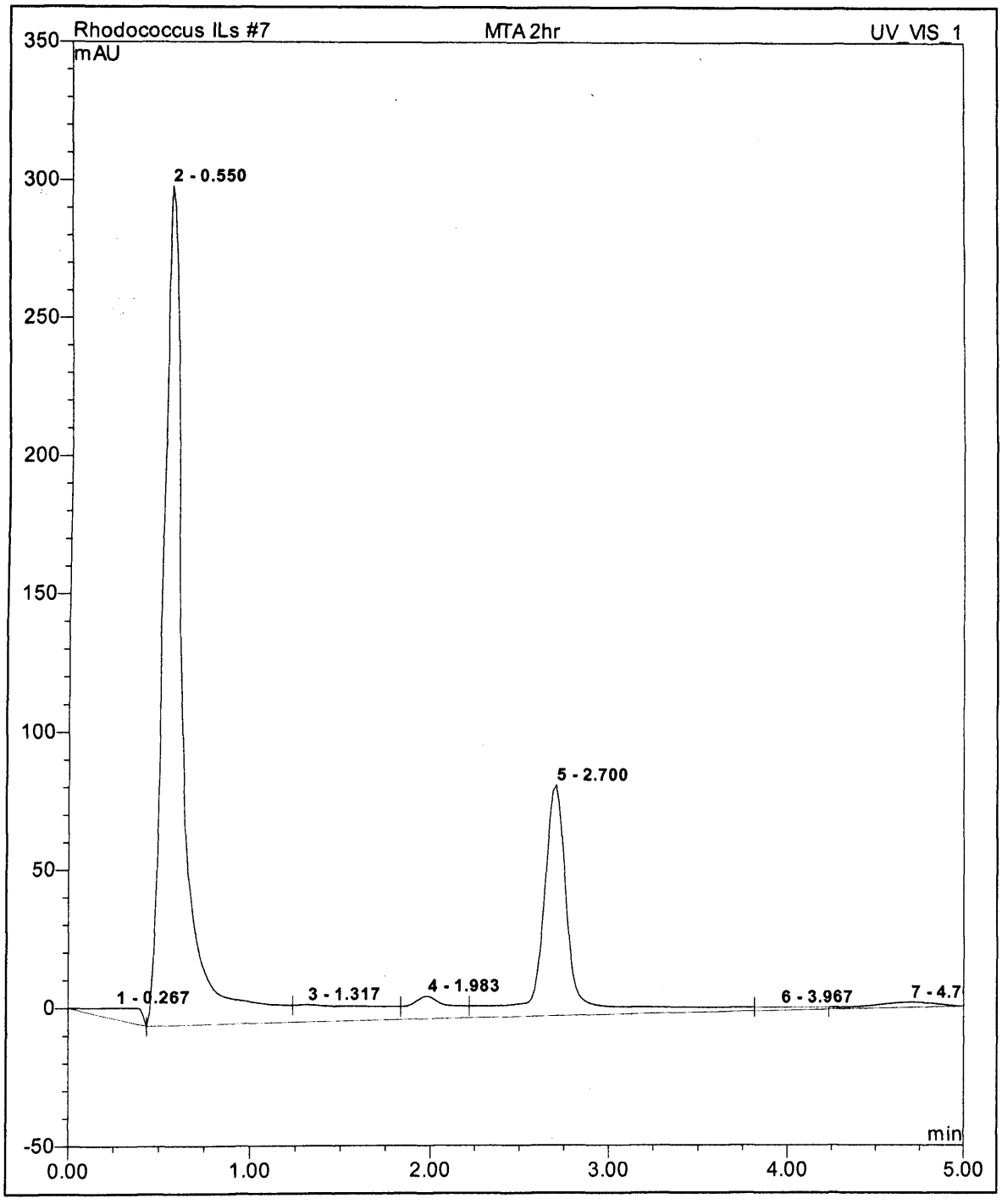

Figure A.2 Sample chromatogram showing separation of 6-Br-β-tetralone eluting at 2.7 minutes and 6-Br-β-tetralol eluting at 1.98 minutes. Samples were analysed as described in Section 2.8.1 on a short column.

A.2 HPLC analysis of the bioreduction of 4'Br-2,2,2-trifluoroacetohphenone to 4'-Br-2,2,2-trifluoroacetophenyl alcohol

A.2.1 Calibration curves

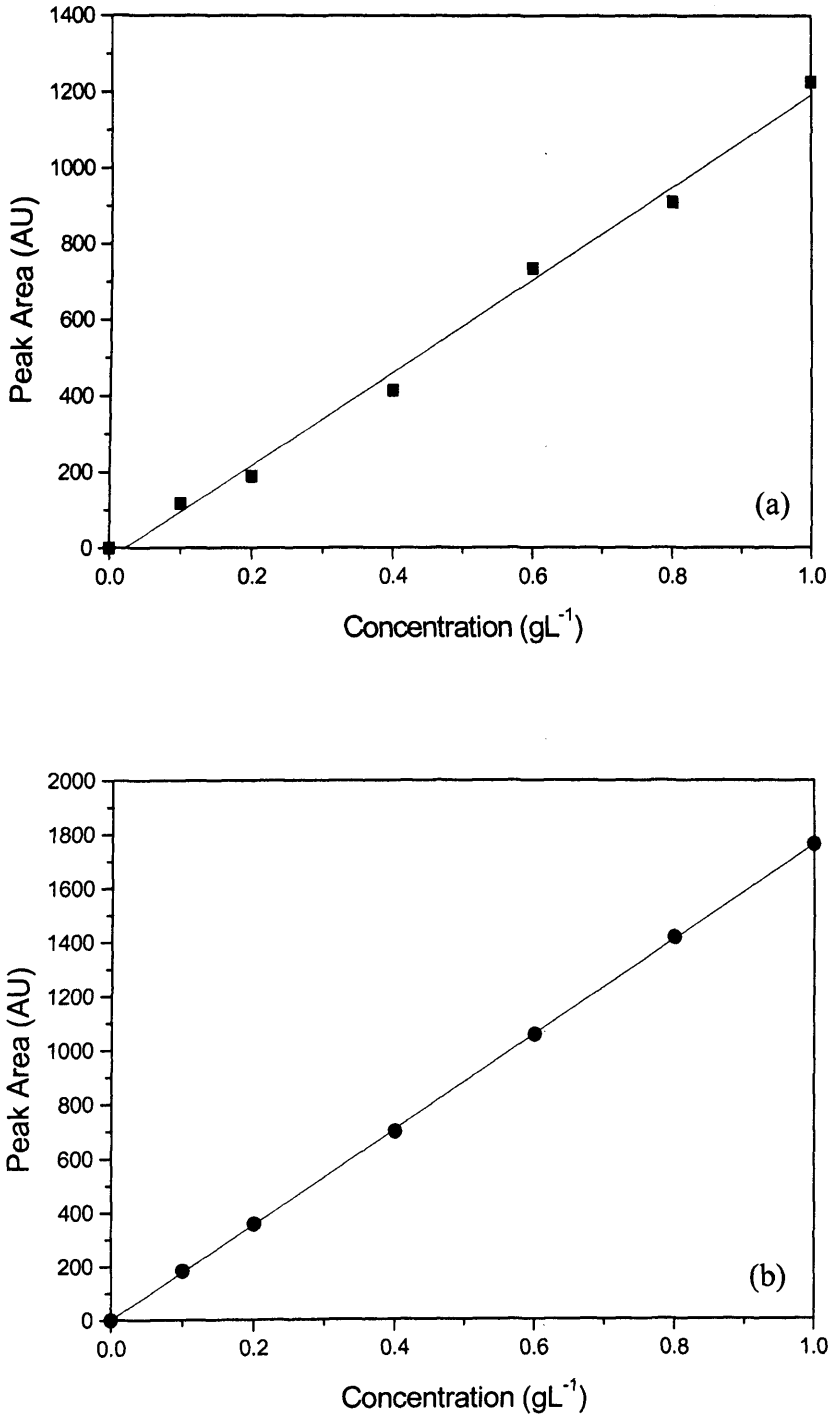

Figure A.3 HPLC calibration curves for (a) 4'-Br-2,2,2-trifluoroacetophenone and (b) 4'Br-2,2,2-trifluoroacetophenyl alcohol. Samples were analysed as described in Section 2.8.1.

A.2.2 Sample chromatogram

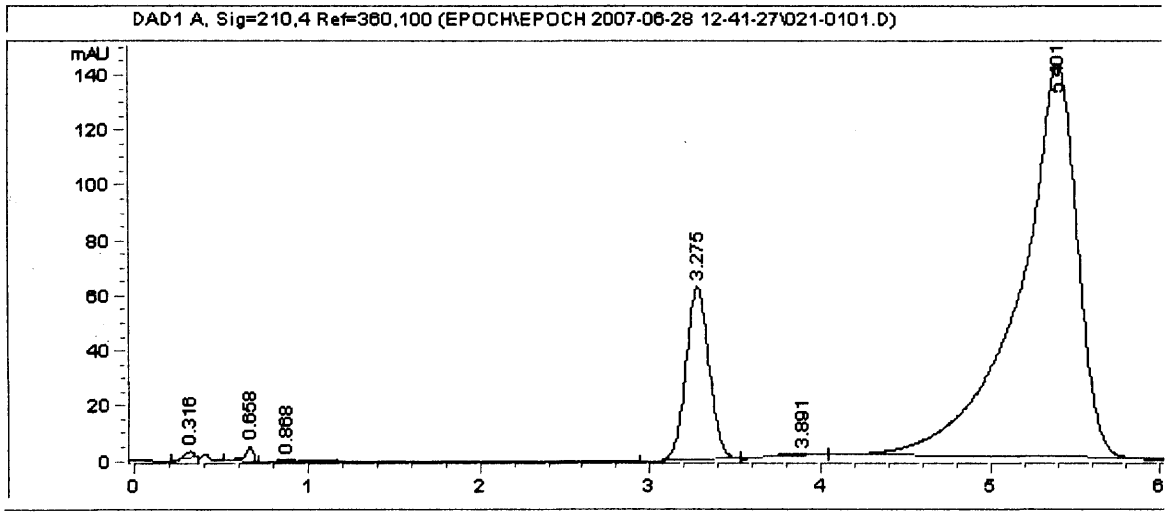

Figure A.4 Sample chromatogram showing separation of 4'Br-2,2,2-trifluoroacetophenone eluting at 5.3 minutes and 4'-Br-2,2,2-trifluoroacetophenyl alcohol eluting at 3.28 minutes. Samples were analysed as described in Section 2.8.1.

A.3 HPLC analysis of the biocatalytic oxidation of 1-indanone to 3,4-dihydrocoumarin

A.3.1 Calibration curves

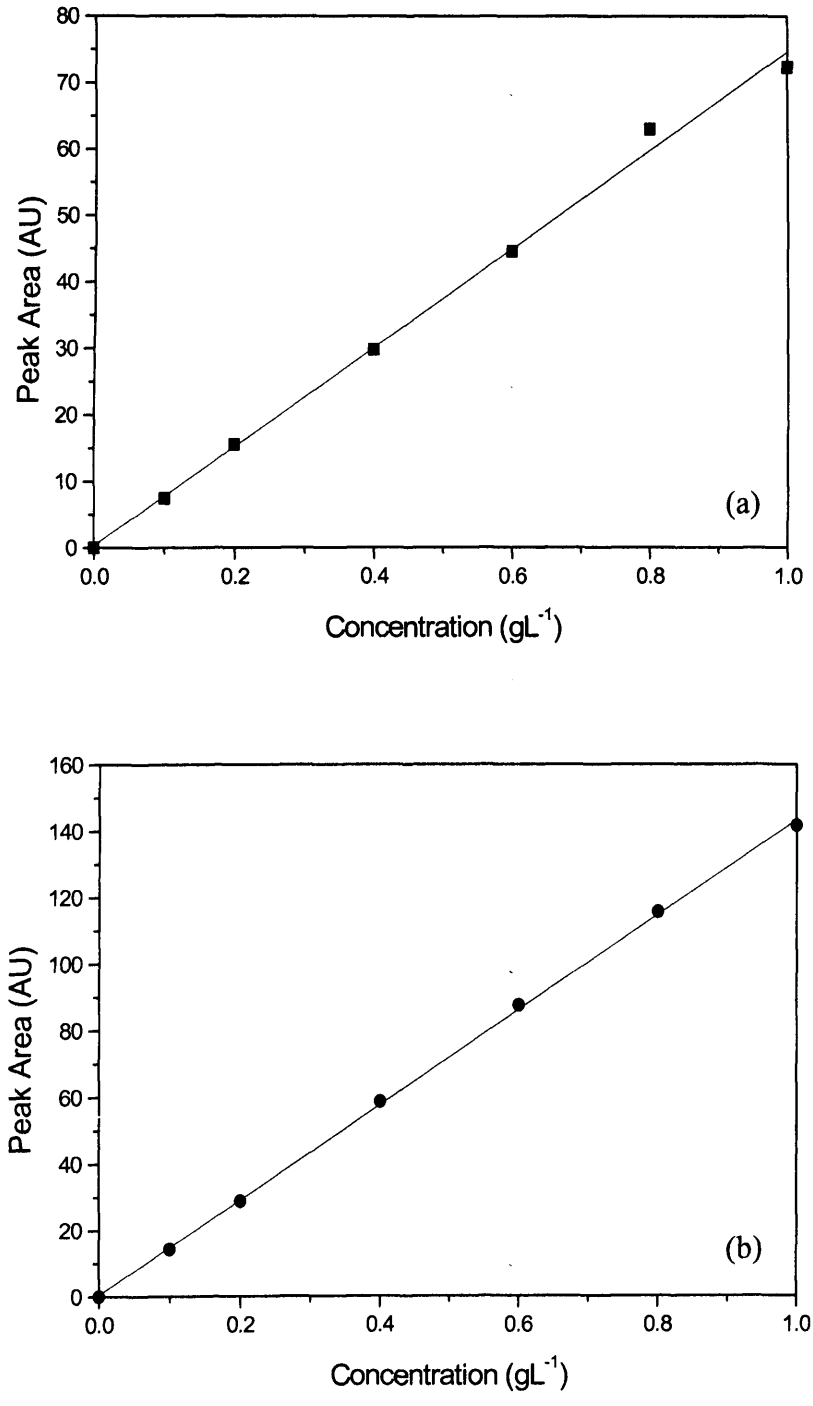

Figure A.5 HPLC calibration curves for (a) 1-indanone and (b) 3,4-dihydrocoumarin. Samples were analysed as described in Section 2.8.1.

A.3.2 Sample chromatogram

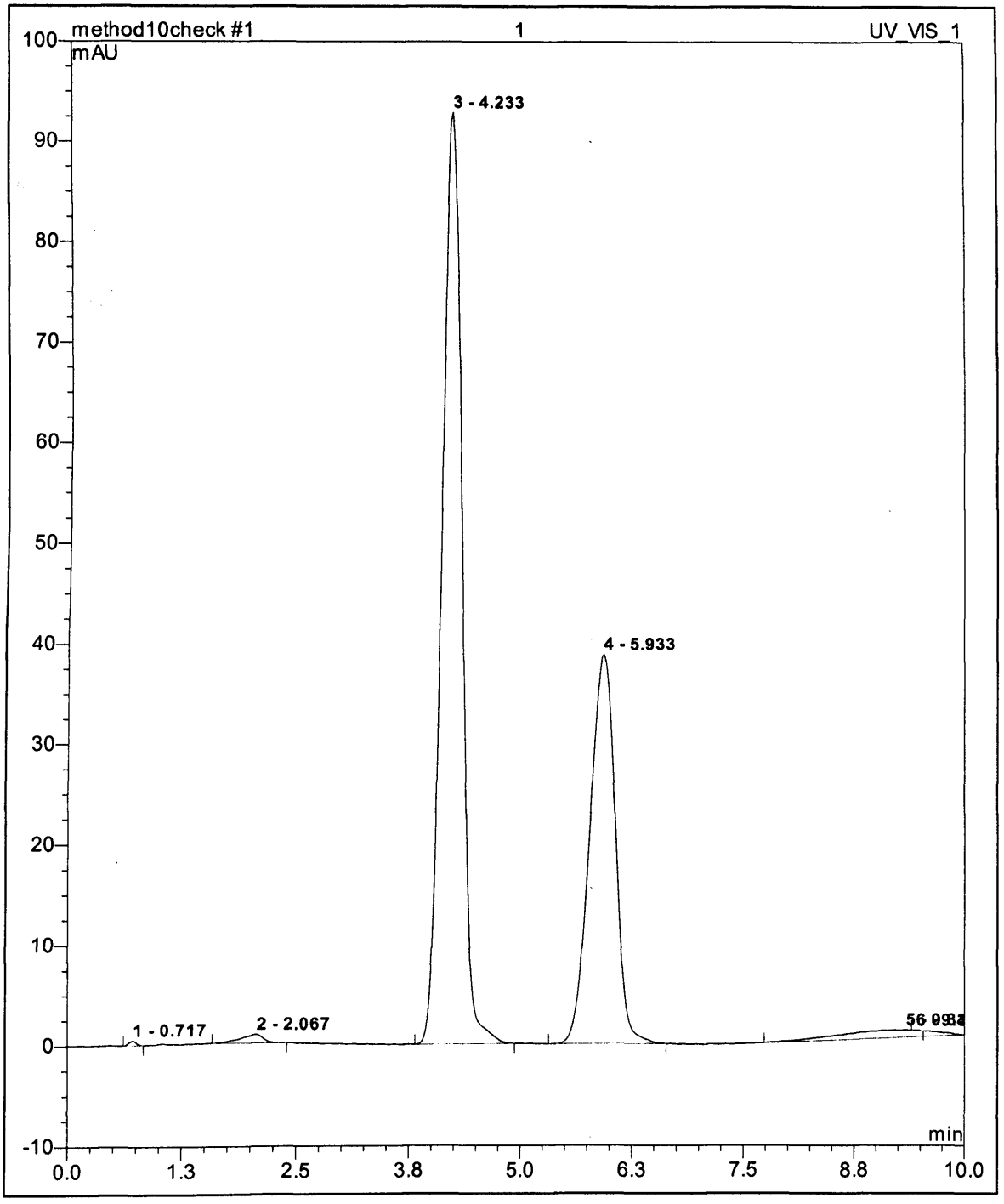

Figure A.6 Sample chromatogram showing separation of 1-indanone eluting at 5.93 minutes and 3,4-dihydrocoumarin eluting at 4.23 minutes. Samples were analysed as described in Section 2.8.1.

Appendix B: Whole cell bioreduction of 6-Br-β-tetralone by *T. capitatum* MY1890

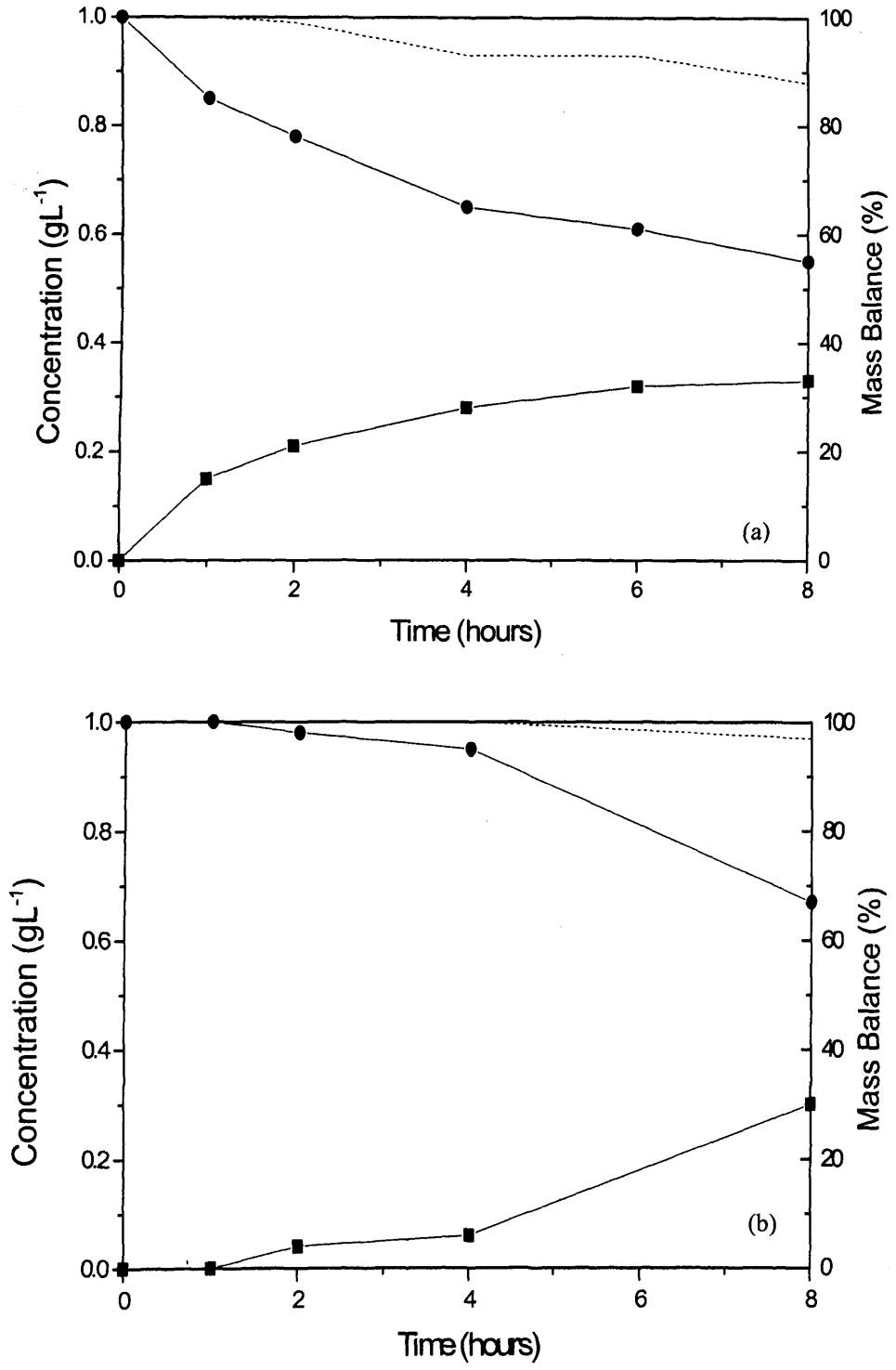

Figure B.1 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the yeast *T. capitatum* MY1890 in the presence of 20% v/v (a) [Bmim][BF_4]; (b) [CABHEM][$MeSO_4$]: (■) 6-Br-β-tetralone; (●) 6-Br- β-tetralol; (-) mass balance. Cultivation was performed as described in Section 2.6. Bioconversion was performed as described in Section 2.7.1 and the reaction was monitored by HPLC analysis as described in Section 2.8.1.

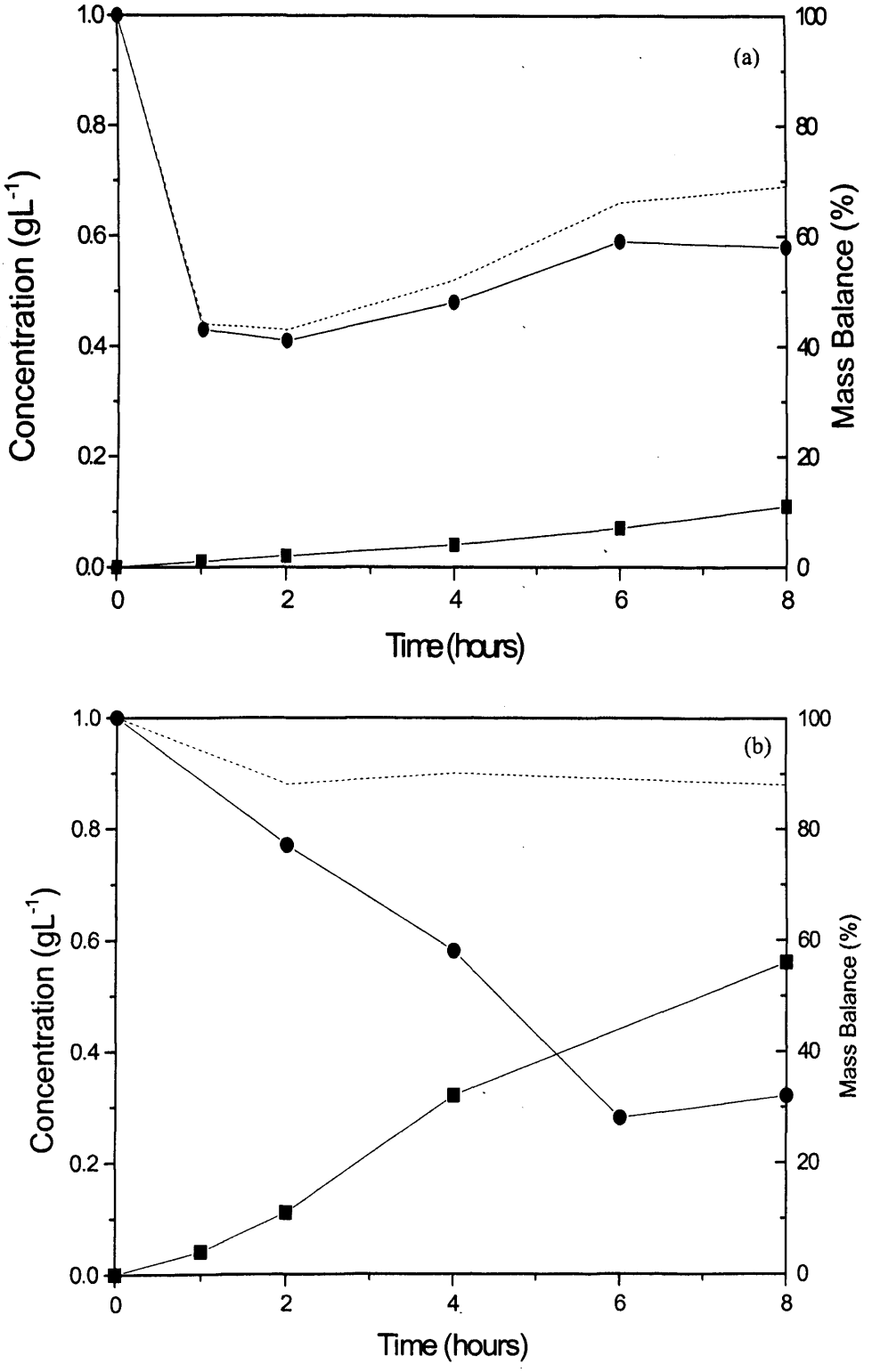

Figure B.2 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the yeast *T. capitatum* MY1890 in the presence of 20% v/v (a) [Bmim][$MDEGSO_4$]; (b) [Oc_3MeN][NTf2]: (■) 6-Br-β-tetralone; (●) 6-Br- β-tetralol; (-) mass balance. Cultivation was performed as described in Section 2.6. Bioconversion was performed as described in Section 2.7.1 and the reaction was monitored by HPLC analysis as described in Section 2.8.1.

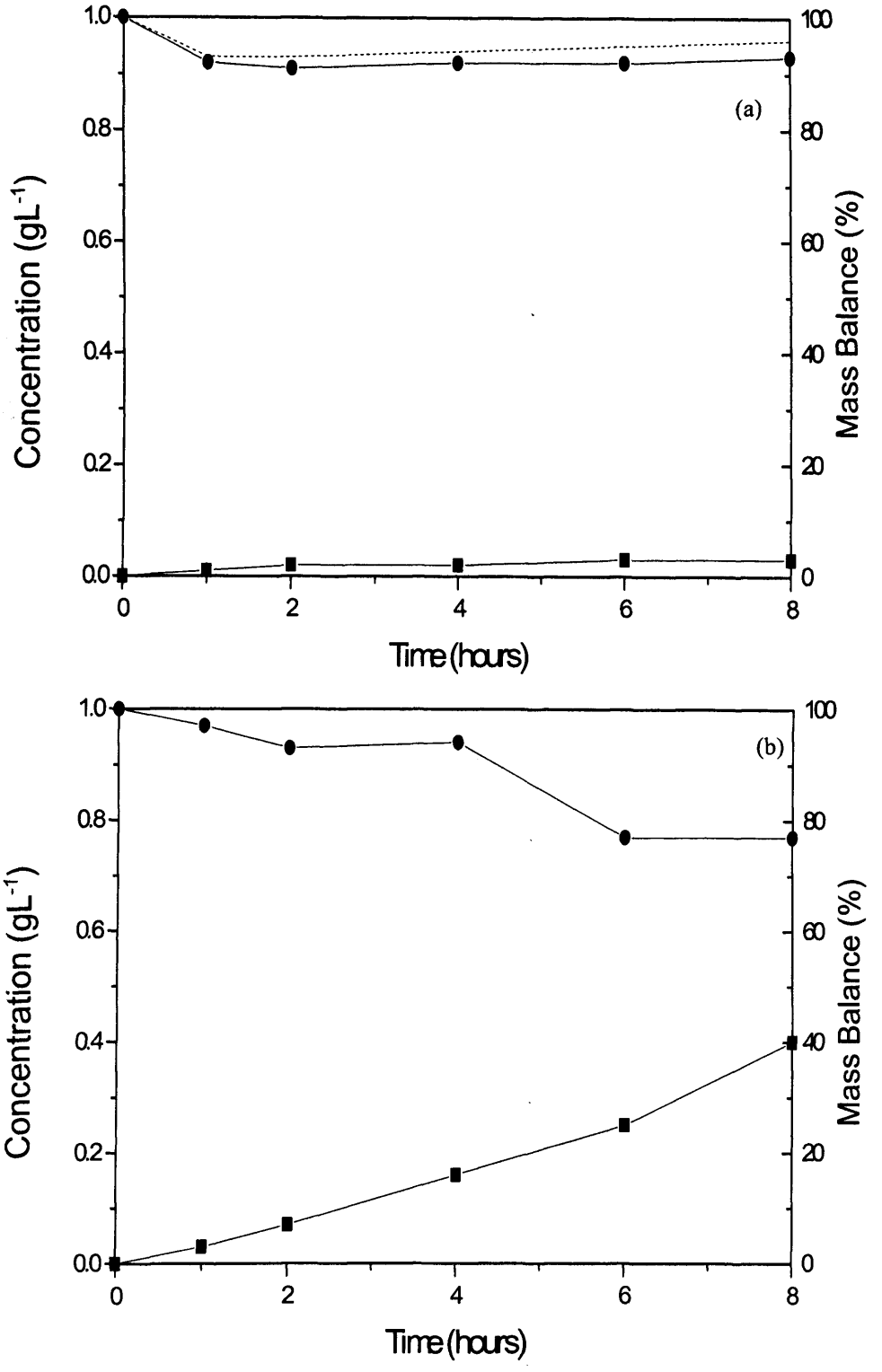

Figure B.3 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the yeast *T. capitatum* MY1890 in the presence of 20% v/v (a) [Bmim][$OcSO_4$]; (b) [Bmim][PF_6]: (■) 6-Br-β-tetralone; (●) 6-Br- β-tetralol; (-) mass balance. Cultivation was performed as described in Section 2.6. Bioconversion was performed as described in Section 2.7.1 and the reaction was monitored by HPLC analysis as described in Section 2.8.1.

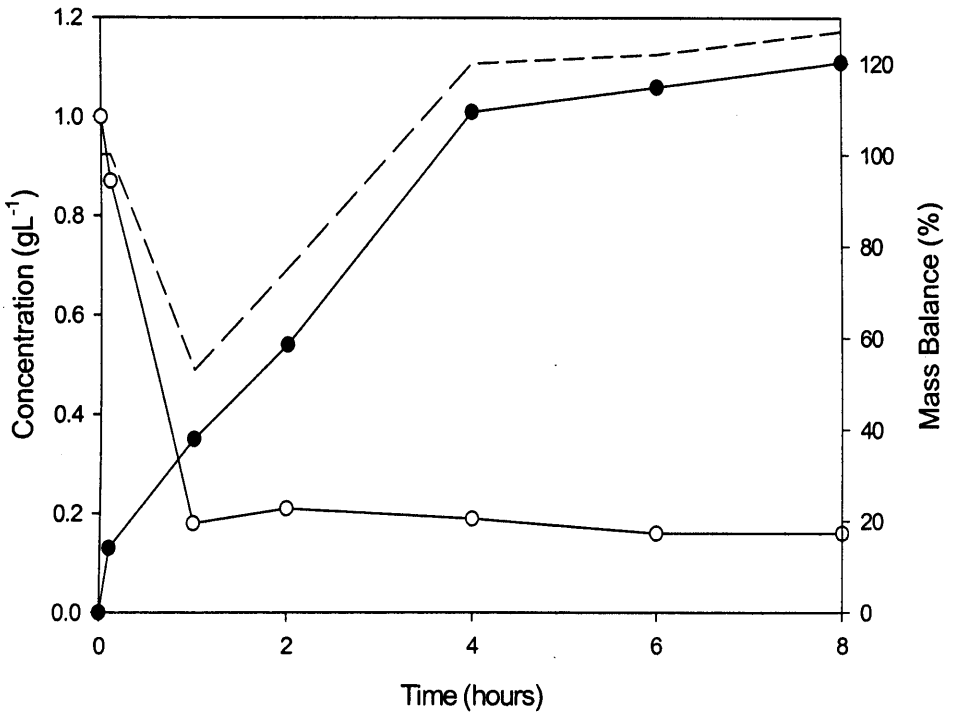

Figure B.4 Time course of the bioreduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by the yeast *T. capitatum* MY1890 in the presence of 20% v/v [Emim][TOS]: (●) 6-Br-β-tetralone; (○) 6-Br- β-tetralol; (-) mass balance. Cultivation was performed as described in Section 2.6. Bioconversion was performed as described in Section 2.7.1 and the reaction was monitored by HPLC analysis as described in Section 2.8.1.

Appendix C: Flow cytometry analysis of *R. erythropolis* MA7213 in a range of co-solvents.

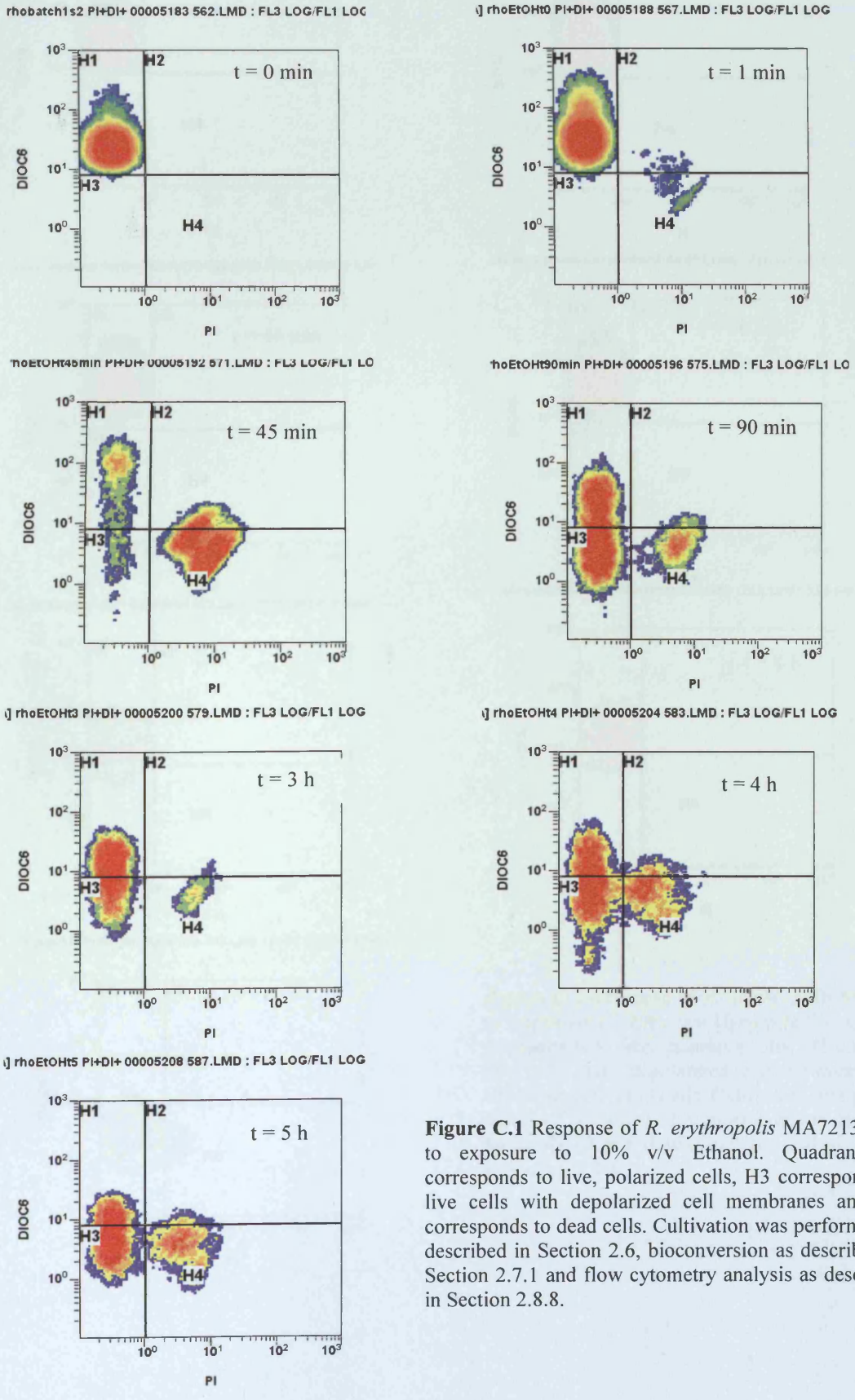

Figure C.1 Response of *R. erythropolis* MA7213 cells to exposure to 10% v/v Ethanol. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

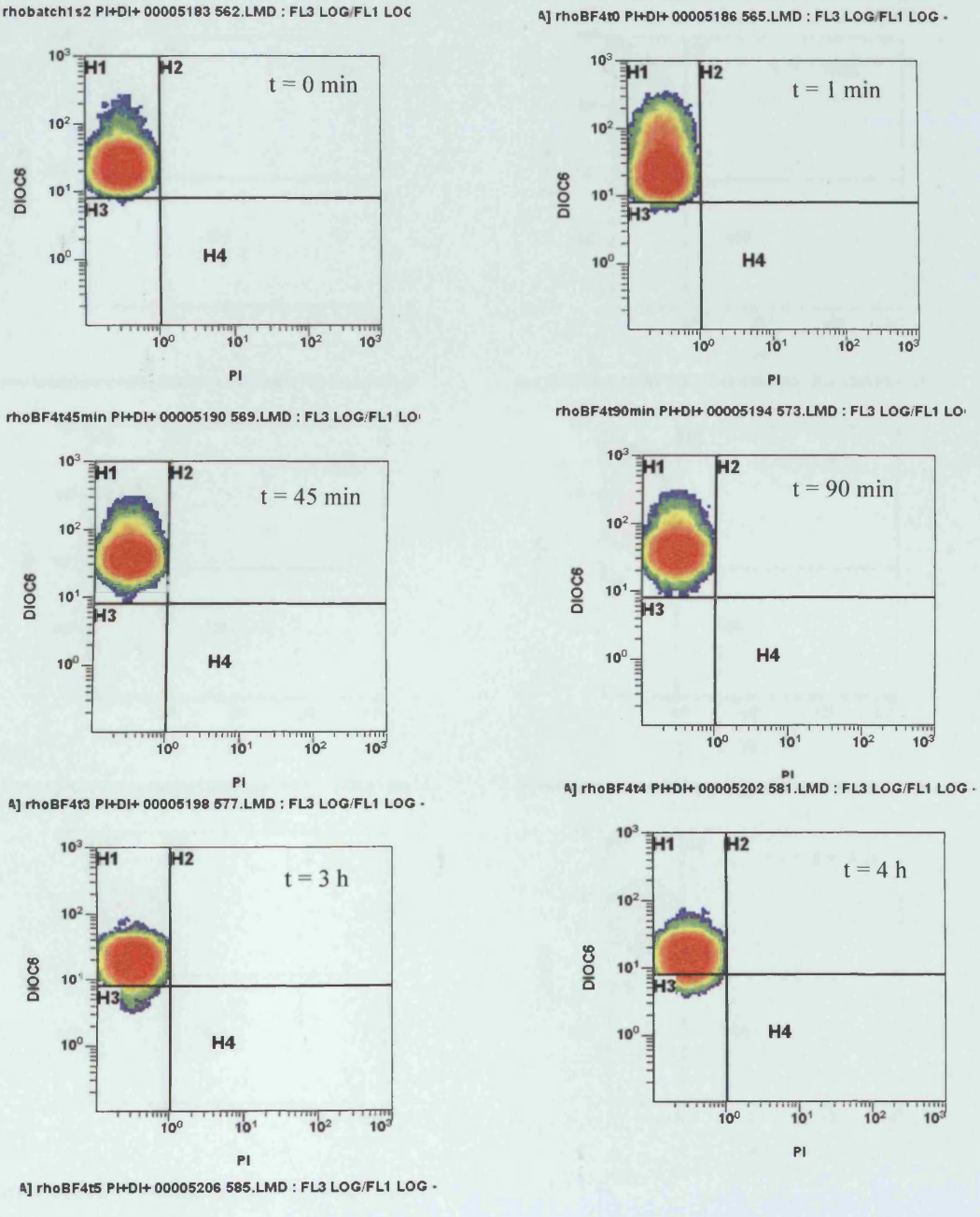

Figure C.2 Response of *R. erythropolis* MA7213 cells to exposure to 20% v/v [Bmim][BF_4]. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

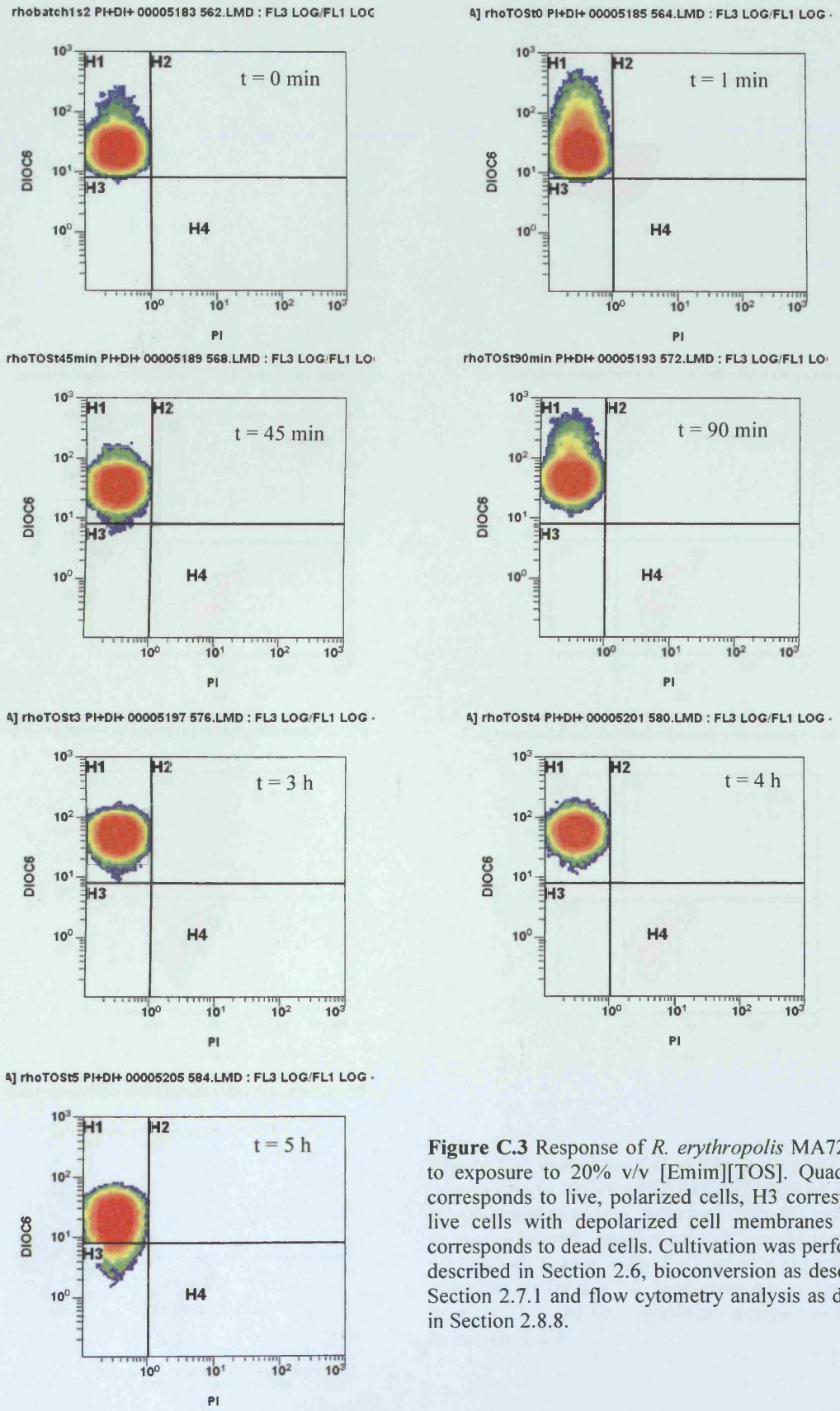

Figure C.3 Response of *R. erythropolis* MA7213 cells to exposure to 20% v/v [Emim][TOS]. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

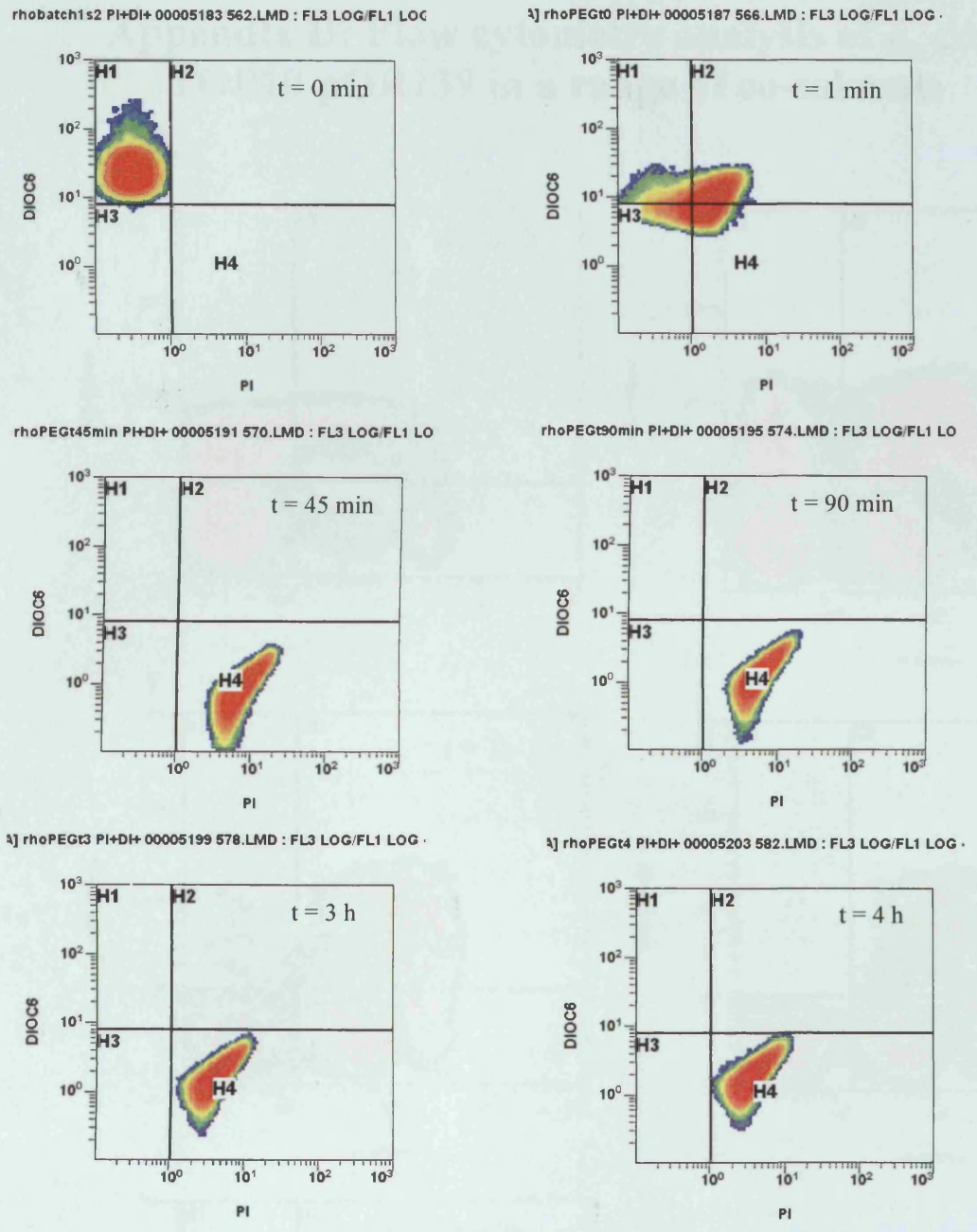

Figure C.4 Response of *R. erythropolis* MA7213 cells to exposure to 20% v/v [CABHEM][$MeSO_4$]. Quadrant H1 corresponds to live, polarized cells, H3 corresponds to live cells with depolarized cell membranes and H4 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Appendix D: Flow cytometry analysis of *E. coli* TOP10 pQR239 in a range of co-solvents

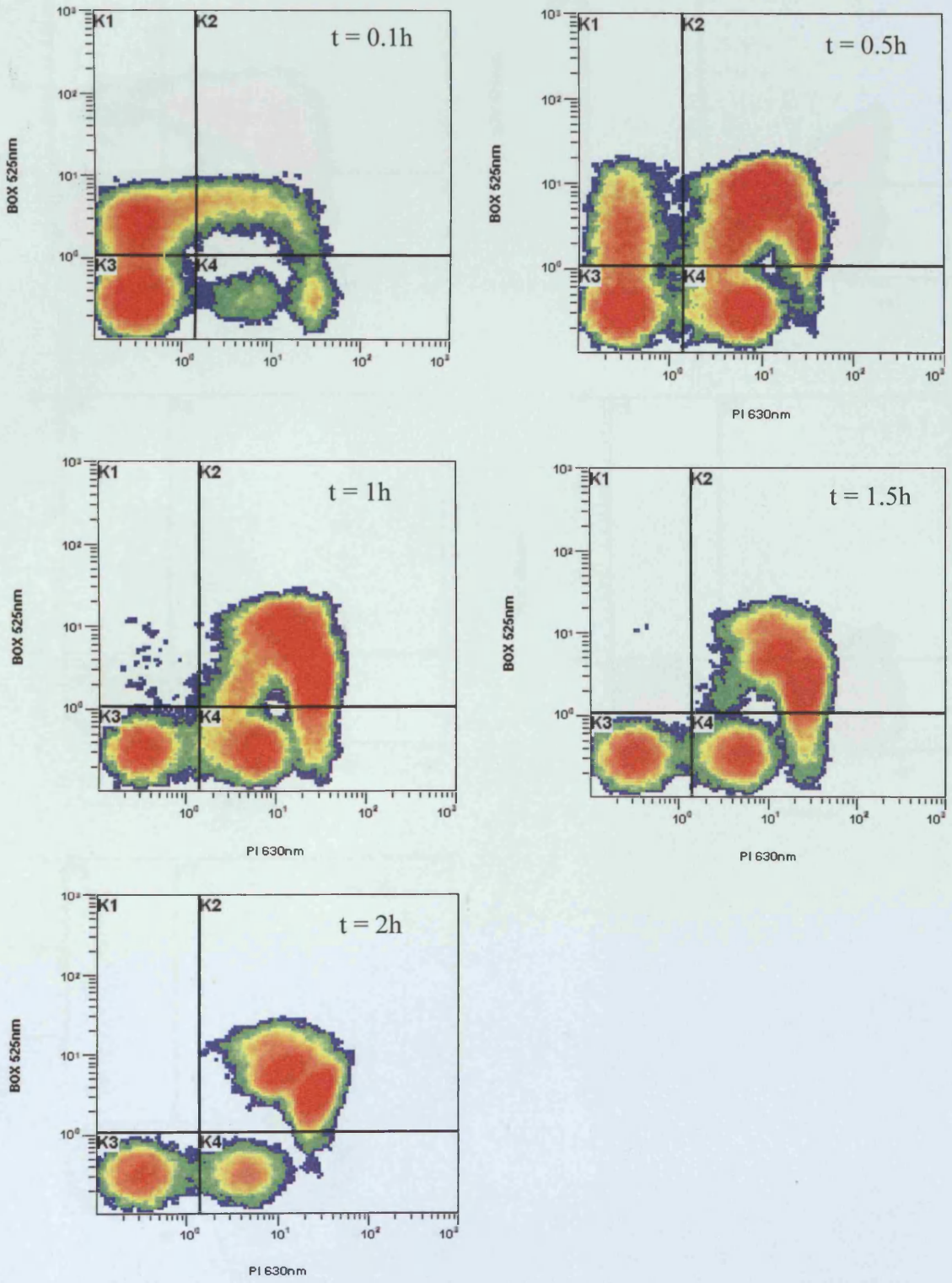

Figure D.1 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Bmim][BF_4]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

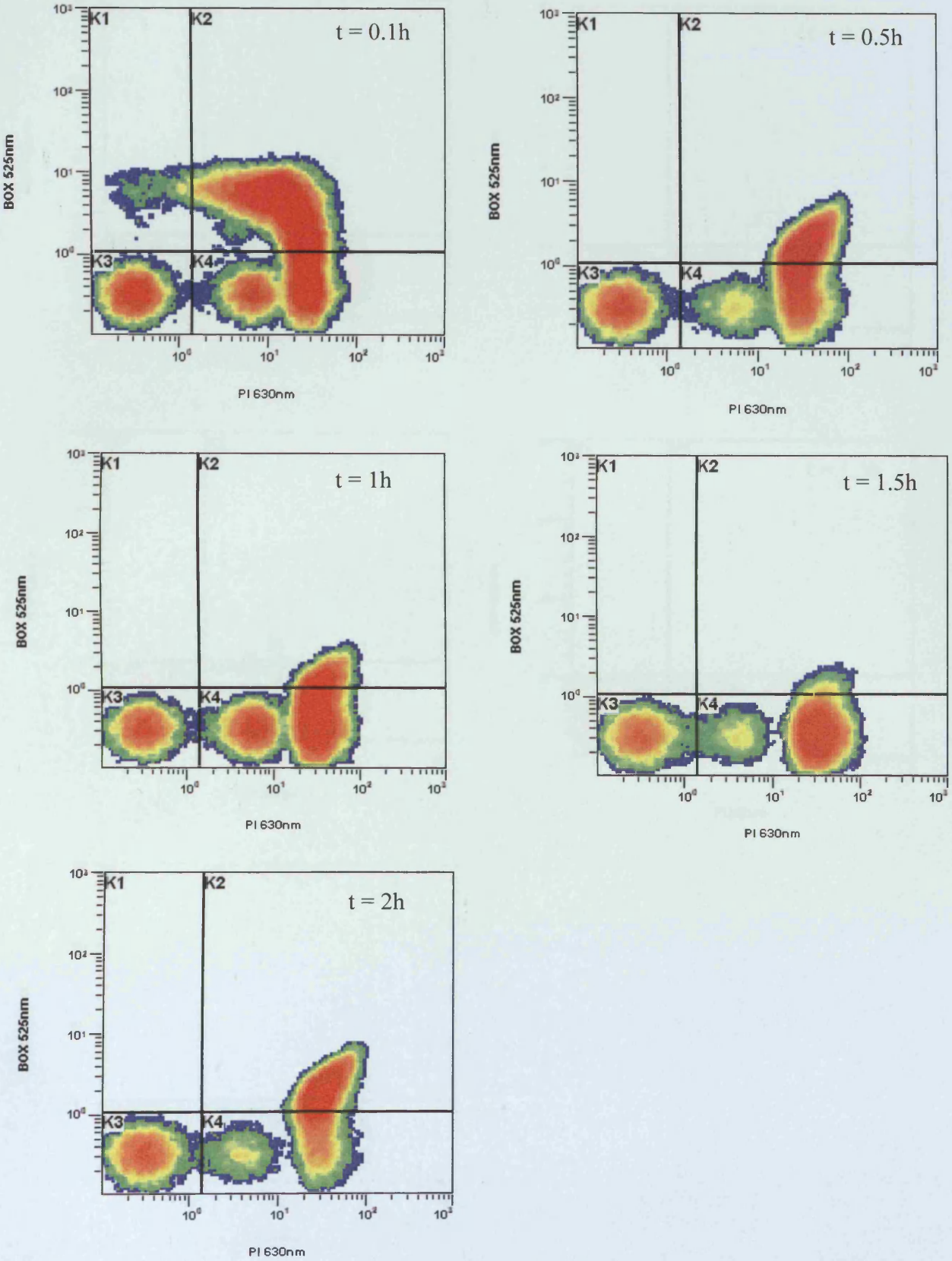

Figure D.2 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Bmim][$MDEGSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

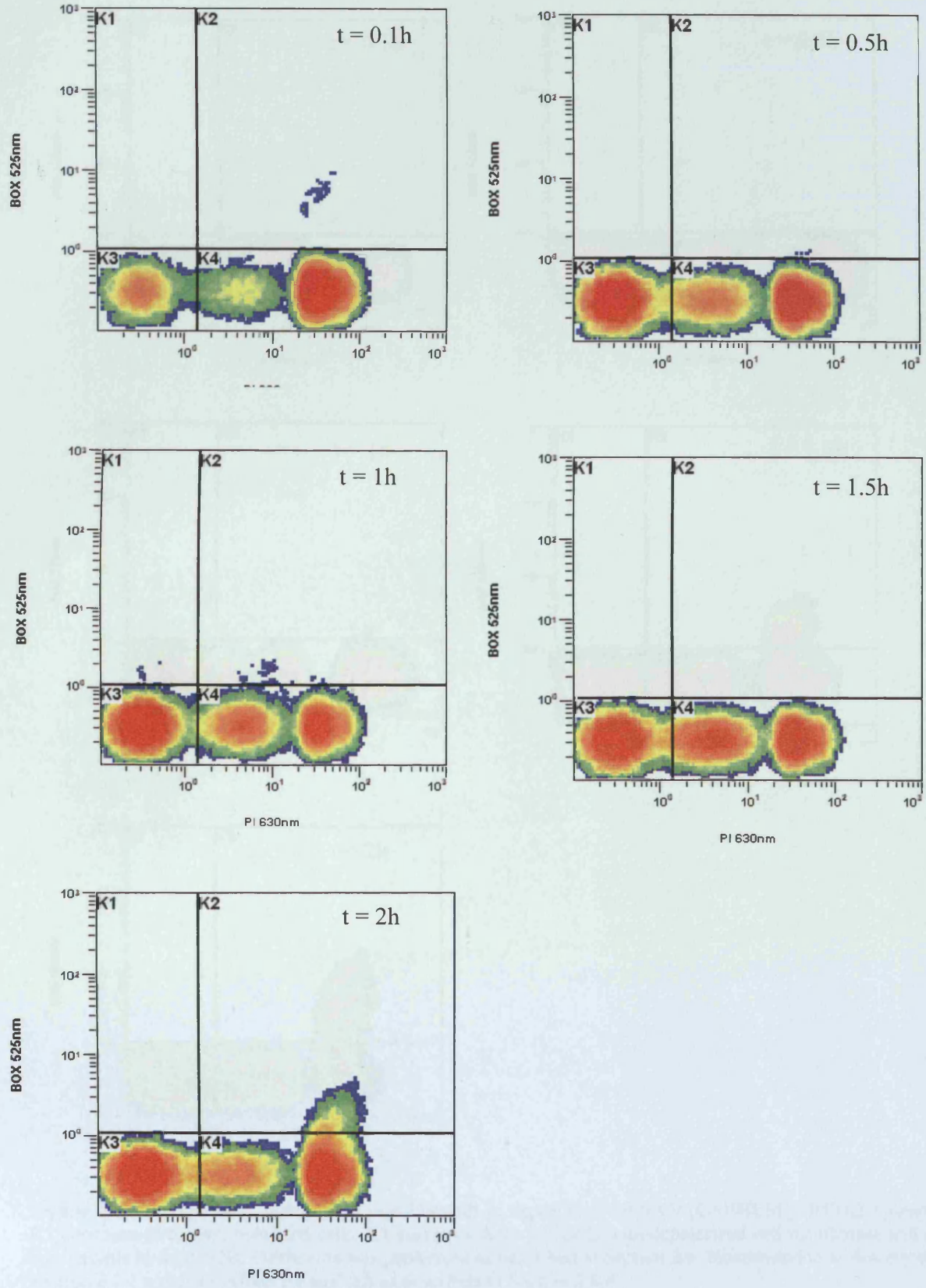

Figure D.3 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Bmim][$OcSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

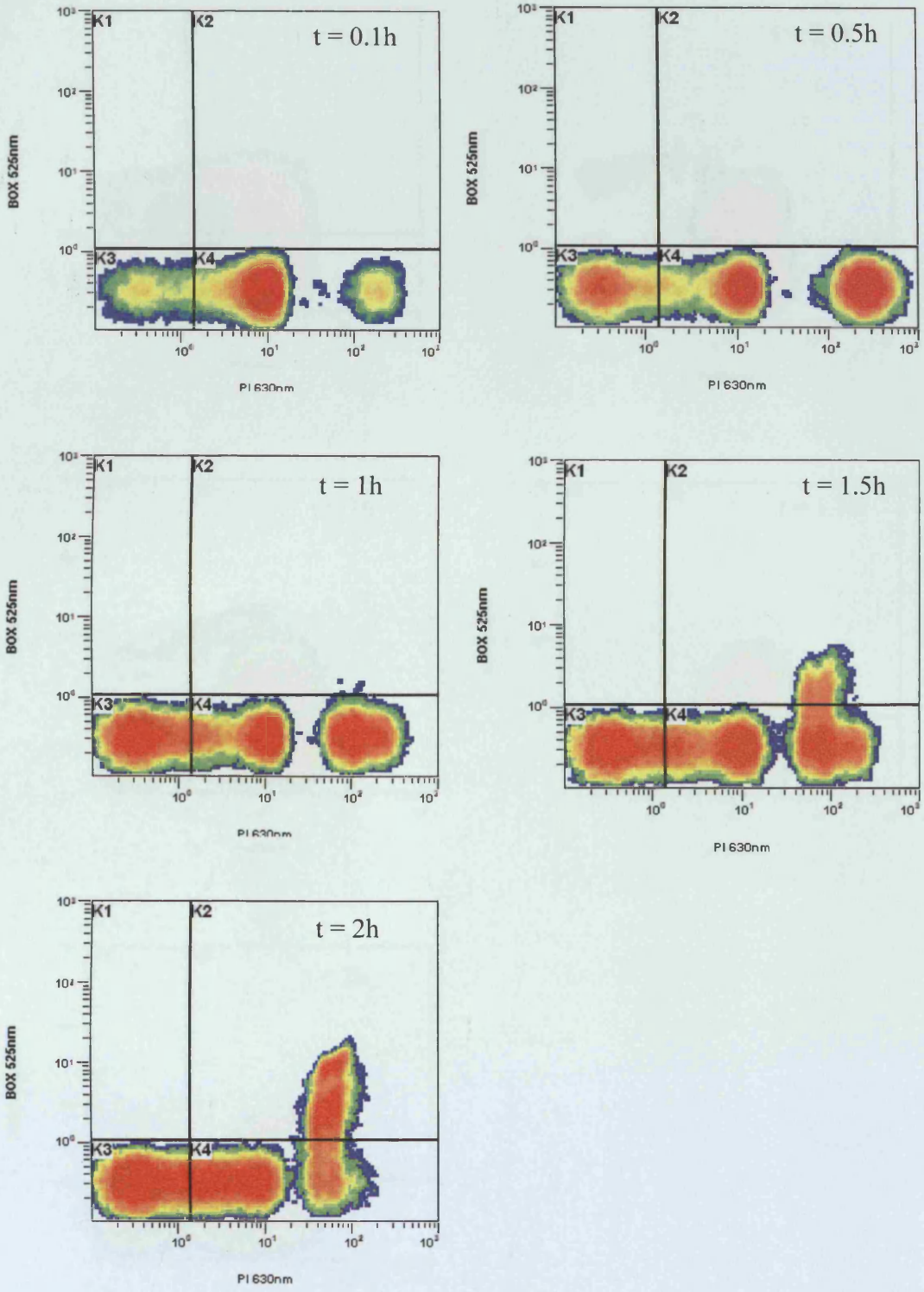

Figure D.4 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [CABHEM][$MeSO_4$]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

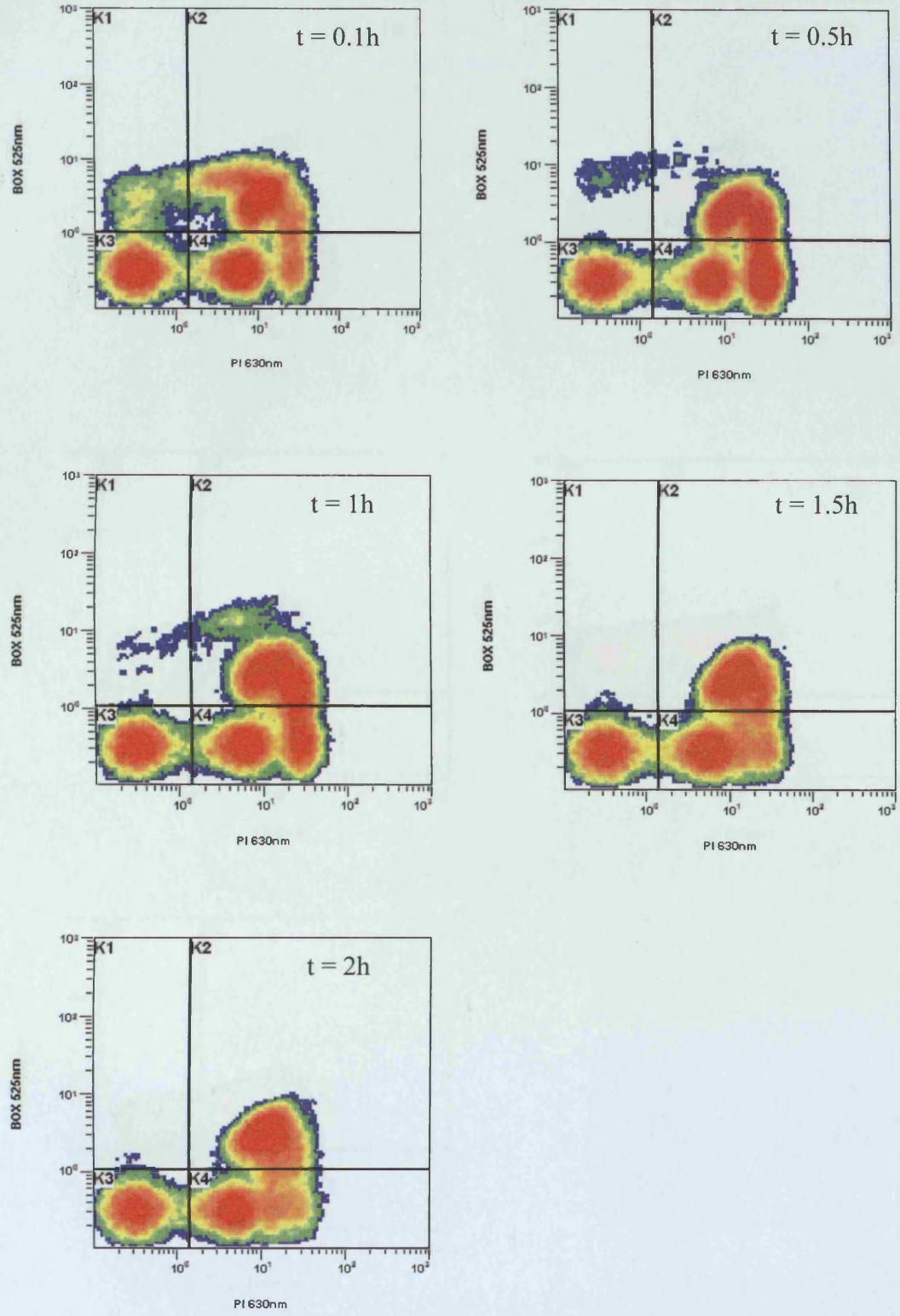

Figure D.5 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Emim][TOS]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

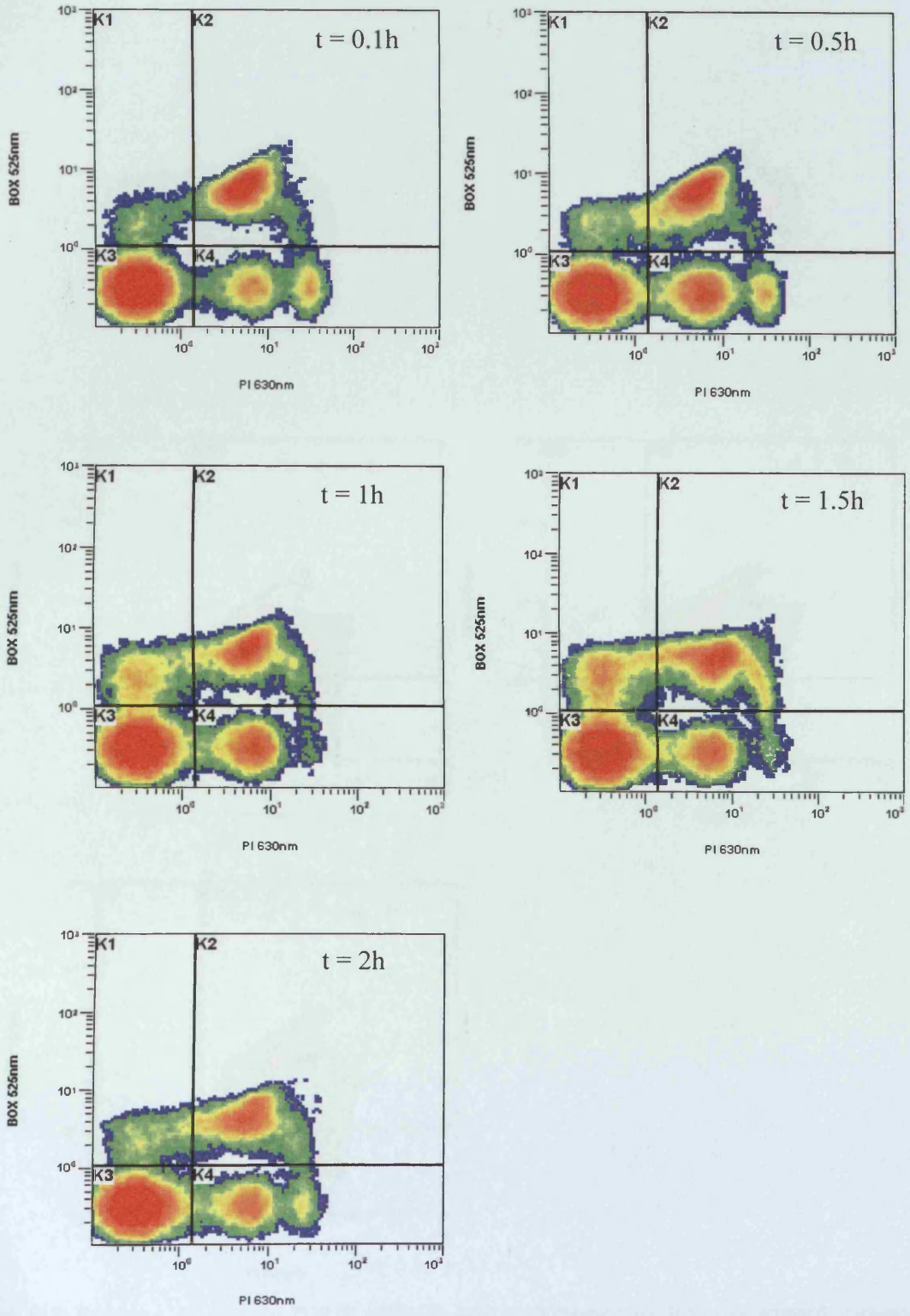

Figure D.6 Response of *E. coli* TOP10 pQR239 cells to exposure to 20% v/v [Oc_3MeN][NTf2]. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

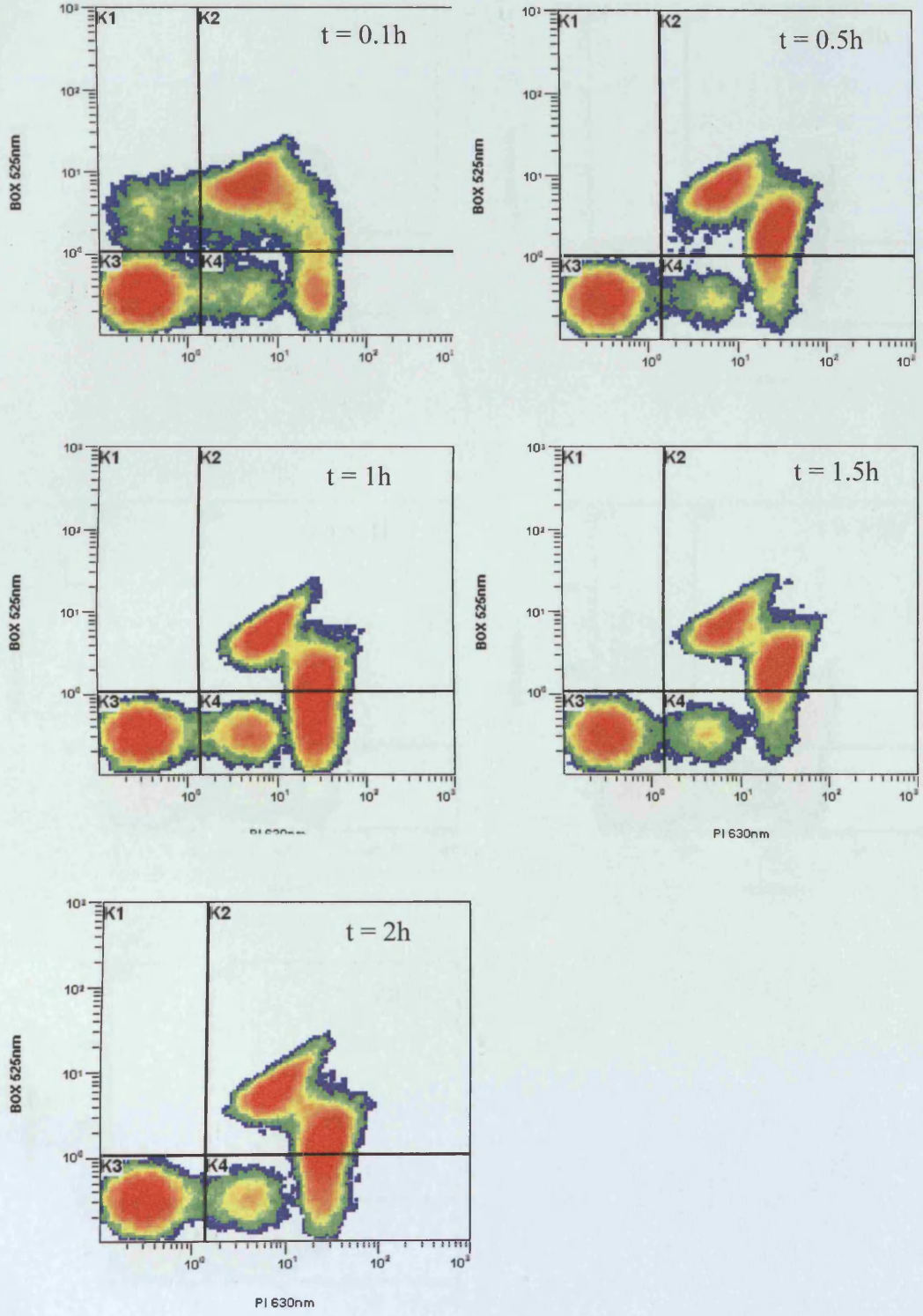

Figure D.7 Response of *E. coli* TOP10 pQR239 cells to exposure to 10% v/v ethanol. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

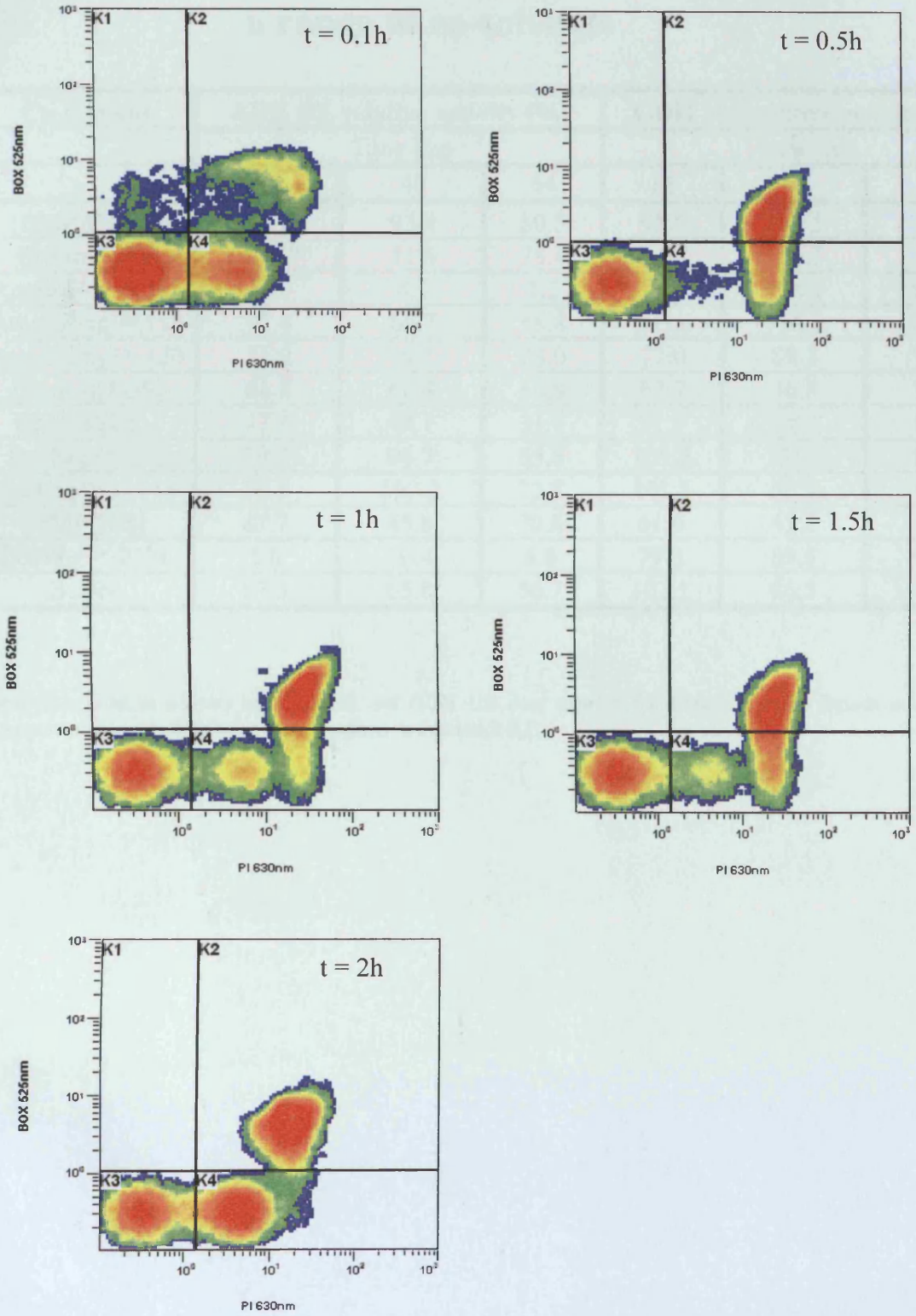

Figure D.8 Response of *E. coli* TOP10 pQR239 cells to exposure to 10% v/v toluene. Quadrant K3 corresponds to live, polarized cells, K1 corresponds to live cells with depolarized cell membranes and K2 corresponds to dead cells. Cultivation was performed as described in Section 2.6, bioconversion as described in Section 2.7.1 and flow cytometry analysis as described in Section 2.8.8.

Appendix E: Stability of ADH RE and GDH 103 in a range of co-solvents

Co-Solvent	ADH RE relative activity (%)			GDH 103 relative activity (%)		
	Time (hr)			Time (hr)		
	15	40	64	15	40	64
[BMP][NTf2]	90.1	93.8	80.5	95.5	111.3	90.6
[Bmim][PF_6]	102.7	71.6	78.4	94.4	101.7	78.7
AmmoEng™ 102	5.8	4.1	1.2	99.8	116.1	85.7
AmmoEng™ 110	97.4	66.7	58.8	105.6	118.6	88.0
AmmoEng™ 120	36.0	29.1	28.0	77.0	89.3	77.9
[Emim][TOS]	61.8	66.4	50.9	62.7	46.8	31.3
[Bmim][BF_4]	47.4	48.1	31.1	91.7	106.7	82.4
EcoEng™ 212	79.7	96.7	65.8	101.3	121.6	86.9
EcoEng™ 1111P	90.1	102.2	72.8	101.3	90.5	66.1
[EMP][ES]	87.7	85.8	70.8	61.0	42.1	18.7
EcoEng™ 21M	5.6	10.4	6.6	79.3	89.5	61.8
Buffer	83.3	65.8	56.7	101.1	86.5	72.0

Table E.1 Relative activity of ADH RE and GDH 103 over time in the library of ionic liquids and buffer. Enzyme activity was determined as described in Section 2.8.2.